ฟิล์มพอลิแลกติกแอซิด/ไคโทซาน/พอลิคาโพรแลกโทนสำหรับวัสดุปิดแผล

นางสาววาษิณี บุญคง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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POLYLACTIC ACID/CHITOSAN/POLYCAPROLACTONE FILM FOR WOUND DRESSING

Miss Wasinee Boonkong

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Petrochemistry
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Field of Study	Petrochemistry
Thesis Advisor	Associate Professor Amorn Petsom, Ph.D.
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วาษิณี บุญคง : ฟิล์มพอลิแลกติก/ไคโทซาน/พอลิคาโพรแลกโทนสำหรับวัสดุปิดแผล. (POLYLACTIC ACID/CHITOSAN/POLYCAPROLACTONE FILM FOR WOUND DRESSING) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. อมร เพชรสม, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร.ณัฏฐา ทองจุล, 131 หน้า.

ฟิล์มไคโทซาน/พอลิแลกติกแอซิด/พอลิคาโพรแลกโทน ที่บรรจุยาดอกซิไซคลินและโมโนโซเดียมกลูตาเมต สามารถเตรียมได้จากวิธี modified spontaneous emulsification-solvent diffusion ซึ่งผลจากการศึกษาพบว่าฟิล์มที่เตรียมได้สามารถนำมาใช้เป็นวัสดุที่ใช้ควบคุมการปลดปล่อยยาไปยังอวัยวะเป้าหมายของมนุษย์ที่กำหนด โดยในกรณีนี้จะนำไปใช้กับบาดแผลเปิด จากการศึกษาพบว่าอัตราส่วนของการเตรียมฟิล์มที่ไคโทซาน 60% พอลิแลกติกแอซิด 28% พอลิคาโพรแลกโทน 12% ให้คุณสมบัติที่เหมาะสมสำหรับทำเป็นวัสดุสำหรับปิดแผล และได้นำฟิล์มที่เตรียมได้ไปศึกษาค่าการปลดปล่อยตัวยาดอกซิไซคลินและโมโนโซเดียมกลูตาเมต พบว่าแผ่นฟิล์มซึ่งให้ค่าการปลดปล่อยที่ยาทั้งดอกซิไซคลินและโมโนโซเดียมกลูตาเมตเข้าใกล้ 100% และยังได้ทำการทดสอบฤทธิ์การห้ามเลือดของแผ่นฟิล์มที่เตรียมได้ ซึ่งจากผลการทดลองพบว่านอกจากโมโนโซเดียมกลูตาเมตมีฤทธิ์ช่วยทำให้เลือดแข็งตัวได้เร็วขึ้นแล้ว ยังช่วยทำให้เกิดเส้นใยไฟบริน ซึ่งเป็นปัจจัยหนึ่งที่สำคัญในกระบวนการแข็งตัวของเลือดขึ้นอีกด้วย นอกจากนี้ ยังได้ทำการทดสอบฤทธิ์การต้านเชื้อแบคทีเรียของ *Staphylococcus aureus* ซึ่งเป็นแบคทีเรียแกรมบวก และ *Escherichia coli* ซึ่งเป็นแบคทีเรียแกรมลบ จากผลทดลองการทดสอบการยับยั้งเชื้อแบคทีเรียของฟิล์มไคโทซาน/พอลิแลกติกแอซิด/พอลิคาโพรแลกโทน ที่บรรจุดอกซิไซคลินสามารถยับยั้งเชื้อแบคทีเรียทั้งสองได้ โดยสามารถสังเกตได้จากค่าน้ำหนักเซลล์แห้งของแบคทีเรียที่มีค่าน้อยมากเมื่อเปรียบเทียบกับสารละลายแบคทีเรียเมื่อไม่มีการเติมฟิล์มดังกล่าว

สาขาวิชา ปิโตรเคมี ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

507 38746 23: MAJOR PETROCHEMISTRY
KEYWORDS: CHITOSAN/POLYLACTIC ACID/POLYCAPROLACTONE/
WOUND DRESSING/ BLOOD CLOTTING/ ANTIBACTERIAL ACTIVITY

WASINEE BOONKONG: POLYLACTIC ACID/CHITOSAN /
POLYCAPROLACTONE FILM FOR WOUND DRESSING. ADVISOR:
ASSOC.PROF. AMORN PETSOM, Ph.D., CO-ADVISOR: NUTTHA
THONGCHUL, Ph.D., 133 pp.

Doxycycline and monosodium glutamate (MSG) loaded CHI/PLA/PCL blend film was prepared by the modified spontaneous emulsification-solvent diffusion method. The results in this study clearly revealed that the blend film prepared could be possibly used as a device to deliver the active ingredients to the targeted human organ which in this case was the skin with an opened wound. From the film characteristic test, it was found that the CHI/PLA/PCL blend film containing 60% CHI, 28% PLA, and 12% PCL exhibited the good film properties for making the dressing device. Doxycycline/MSG loaded CHI/PLA/PCL blend film could rapidly deliver both doxycycline and MSG at the high release percentage approaching 100% of the amounts loaded. It was observed that MSG accelerated blood clotting and fibrin formation; thus, it demonstrated the good hemostatic activity. The antibacterial activity of doxycycline loaded CHI/PLA/PCL blend film against the gram positive *Staphylococcus aureus* and the gram negative *Escherichia coli* was also investigated. It was cleared that doxycycline released from the blend film played the crucial role in bacterial inhibition as observed from the lowest bacterial cell dry weight observed when compared with the control bacterial culture or the bacterial cultures with the presence of other film studied.

Field of Study: Petrochemistry Student's Signature .....
Academic Year: 2011 Advisor's Signature .....
Co-advisor's Signature .....

Dedicated to my father

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Finally, I would like to dedicate my thesis to my beloved father who passed away and wish to express my deep sense of appreciation to my beloved mother and family for their inspiration, understanding, great support and encouragement throughout my study, without them, the author would have never been able to achieve this goal.

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LIST OF ABBREVIATIONS AND SYMBOLS

δ	chemical shifts
°C	degree celsius
µg	microgram
µm	micrometer
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
AFS	adhesive failure strength
BD	1,4-butanediol
CH	chitosan
$CHCl_3$	chloroform
CL	ε-caprolactone
cm	centimeter
cm^2	square meter
DAD	dialdehyde dextran
DSC	differential scanning calorimetry
g	gram
GPC	gel permeation chromatography
h.	hour
kV	kilovolt
LA	L-lactide
LMW	low molecular weight
M	molar
mg	milligram
MHz	megaHertz
min.	minute
ml	milli-liter
mm	millimeter
$\overline{M}_n$	molecular weight by number
N	normal
N/cm	Newton per centimeter width
nm	nanometer

OH	hydroxyl group
P(LA-*co*-CL)	poly(lactide-*co*-caprolactone)
PBS	phosphate buffer saline
PCL	poly(ε-caprolactone)
PDI	polydispersity
PLLA	poly(L-lactide)
ppm	parts per million
PTOL	pentaerythritol
ROP	ring-opening polymerization
rpm	rounds per minute
SB	D-sorbitol
SEM	scanning electron microscopy
$Sn(Oct)_2$	stannous(II) 2-ethylhexanoate
T_a	temperature of application
T_b	body temperature
TCH	tetracycline hydrochloride
T_g	glass transition temperature
THF	tetrahydrofuran
UV-VIS	ultraviolet visible
v/v	volume by volume
$\overline{M}_w$	molecular weight average by weight
w/w	weight by weight
µl	micro-liter

CHAPTER I

INTRODUCTION

1.1 Background

Nowadays, biodegradable polymers are used to replace petroleum-based materials because the oil crisis and global environmental pollution or disruption is becoming the serious problems in the world. There are many biodegradable polymers such as polylactic acid (PLA), polyhydroxyalkanoates (PHAs), polycarpolactone (PCL) and polyglycolic acid (PGA). Those biodegradable polymers have wide range applications in the biomedical, textile, and packaging fields. Biodegradable polymers represent a class of extremely useful materials for many biomedical and pharmaceutical applications. For example in recent years drug delivery systems, have taken advantages of biodegradable polymeric matrices. A basic requirement for the use of biomaterials for human therapy is their biodegradability and biocompatibility. Poly(e-caprolactone) (PCL) and polylactic acid (PLA), recognized as biocompatible and biodegradable polyesters, are very promising for controlled drug delivery devices. These biodegradable polymers are often used because they have low toxicity, excellent biocompatibility, and nontoxic cleavage biodegradable to product.

Hemorrhage is the leading cause of death from battlefield trauma and the second leading cause of death after an accident and trauma in the civilian community. An effective method for controlling hemorrhage in forward treatment elements (pre-hospital, non-physician providers) would greatly reduce mortality rate and decrease logistical requirement for casualty care. A topical hemostatic agent must control rapidly flowing, otherwise lethal, large venous or arterial hemorrhage, through a pool of blood without vascular control. Relatively minor injuries, such as a superficially cut finger or scrapped knee, are often covered with sterile cotton gauze pads that are held over the injured site.

by pressure from an adhesive barrier strip affixed to adjacent normal skin. Such first aid strips may be used to sequester small amounts of blood within the absorbent pad until components of blood and damage tissue can form fibrin-based clot. When large vessels are cut or torn, the rapid flow of escaping blood tends to remove fibrin clots before they can clog the vessel and adhere to the adjacent damaged tissue. There is a requirement for material able to arrest such major hemorrhage. In many cases, blood adhering to damage tissue is clotted and slowly transformed into a scab that serves as a skin substitute. The scab retains body fluids while sealing out bacteria and other environmental hazards. Wound healing normally takes place under cover of the protective scab, which prevents drying of underlying cells and undesirable inflammatory reactions which limit normally healing. Such healing requires closure of any void with fibroblasts and the migration of epidermal cells over fibroblasts and fibroblastic collagen products under physiological conditions.

There is a need for methods able to rapidly provide the protective functions normally provided by epithelial cells and to foster re-epithilialization for re-establishment of such functions. An artificial scab is expected to provide such immediate protection. Polymers in forms of aqueous gel, mucilage, glue, cream, granule, and particle, such as polyvinyl alcohol, polyvinyl acetate, polyphosphate, poly-4-hydroxybutyrate, and chitosan, are now commercially available to prevent trauma bleeding by providing the template for scab formation. Polyvinyl alcohol and polyvinyl acetate in form of gel helped cover and adhere to the open wound, denuded tissue, or burned skin so that bleeding or fluid loss was reduced or stopped. Not only immediate clotting, biocompatibility and antimicrobial activity are also the requirements in treatment of hemorrhage. Biomaterials such as polylactic acid (PLA) and chitosan can provide such the required properties. PLA is currently used in a number of biomedical applications, such as sutures, stents, dialysis media, and drug delivery devices. It is also being evaluated as a material for tissue engineering. While chitosan's properties allow it to rapidly clot blood, and has recently gained approval in the US for use in bandages and other hemostatic agents. In addition, chitosan is

hypoallergenic, and has natural antibacterial properties, further supporting its use in bandages **(**Ahuja et al., 2007**).**

Therefore, in this work, polylactic acid will be synthesized by direct polycondensation. Polycaprolactone, blend of PLA and PCL and blended PLA/PCL/chitosan film will be prepared. Later, the films will be used to construct a biodegradable device for controlled delivery of active ingredient with hemostatic activity to speed up blood clotting and at the same time providing the network where fibrin-based clot form. The expected outcome of this research is the manufacturing process of the biodegradable device for treatment of hemorrhage. With further in vivo study on animal and clinical trial, it is expected that this biodegradable device can be of commercial interest.

1.2 Research Objectives and Scope of the Study

The goal of this work was to tailor a biodegradable wound dressing device containing active ingredient to rapidly control hemorrhage. To achieve the goal of this work, four specific objectives were studied.

1. To synthesize polylactic acid by polycondensation.
2. To characterize the resulting polymer by ^{1}H NMR, GPC and DSC.
3. Preparation of CHI/PLA/PCL blended film for wound dressing device.
4. *In vitro* study on blood clotting process using blood clotting promoting agent.
5. Evaluation of antibacterial activity.

The scope of this work is summarized in Figure 1.1.

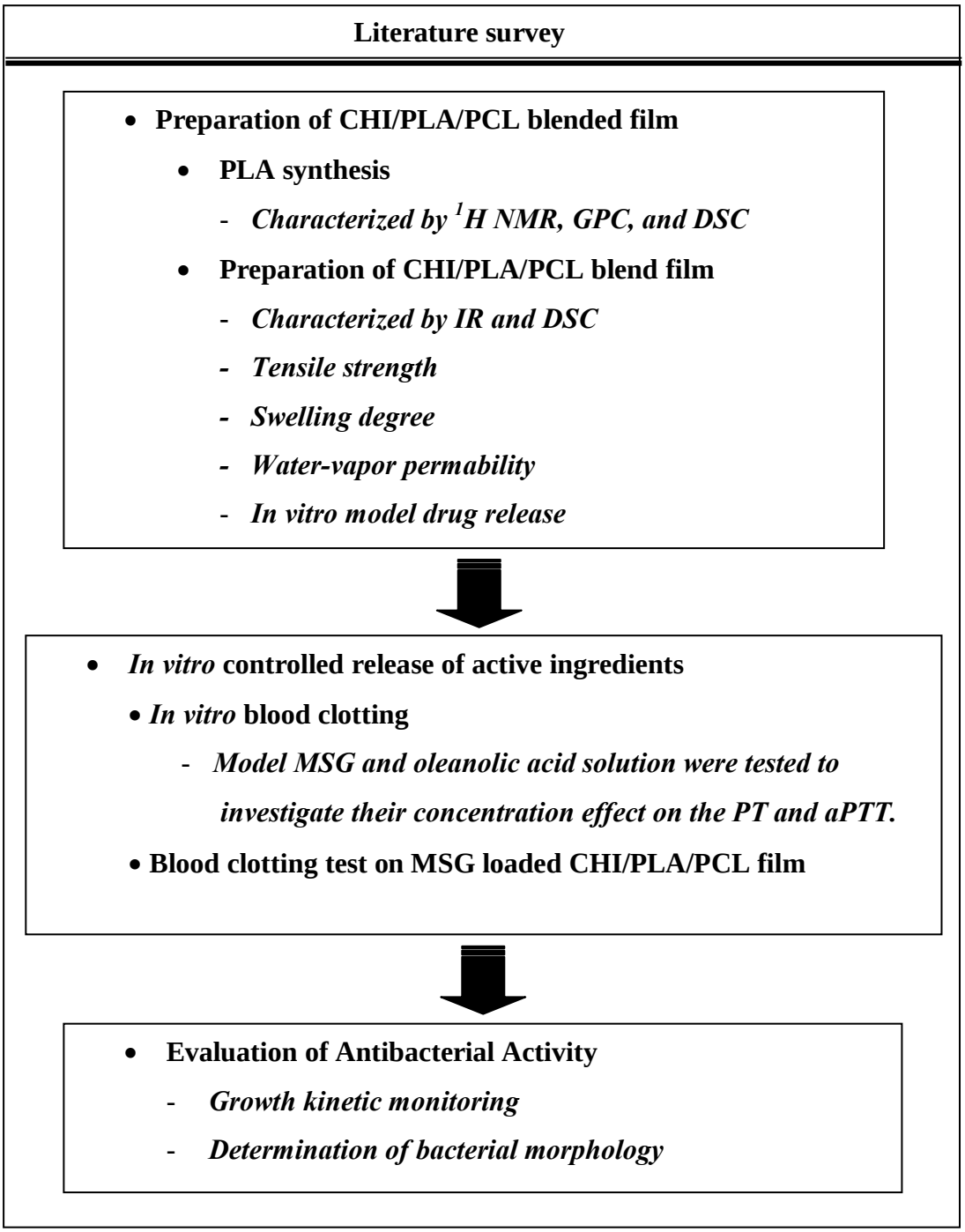

Figure 1.1 Scope of research

CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Polylactic acid synthesis by direct polycondensation

PLA is a biodegradable, thermoplastic, aliphatic polyester derived from renewable resources, such as cornstarch or sugarcanes. The synthetic routes to obtain PLA are basically through direct condensation of the free acid or ring opening polymerization of acid esters.

The direct condensation of PLA from L-lactic acid is shown in figure 2.1.

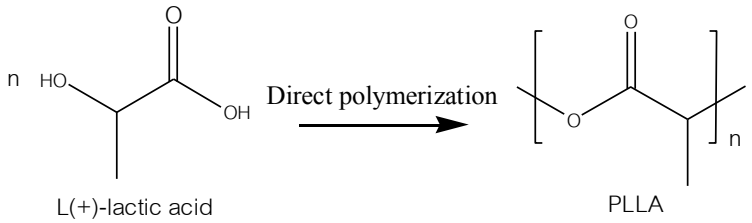

Figure 2.1 PLA polymerization by direct polymerization from L-lactic acid

In 1995, Ajioka *et al.* studied the direct polymerization process of PLA. Mw higher than 300,000 was obtained by direct condensation polymerization of lactic acid in the presence of stannous octoate as catalyst and organic solvent is lauryl alcohol.

In 2003, Dutkiewicz *et al.* studied the effect of solvent, o-dichlorobenzene, p-xylene, o-chlorotoluene, and diphenyl ether and pressure on direct polycondensation of PLA. PLA with the highest molar mass was obtained in the synthesis carried out with the presence of diphenylether under vacuum.

In 2009, Achmad *et al.* the activation energy (E_a) of direct polymerization of PLA was which was investigated by found to be larger than that required by catalyzed ring opening polymerization (ROP). The maximum PLA molecular weight obtained

from direct polycondensation under vacuum without catalysts, solvents and initiators was 90 kDa at 200 °C after 89 hours.

The ring opening polymerization of PLA is a multistep process. The first step is linear polycondensation of L-lactic acid to low molecular weight of poly (L-lactic acid), this reaction as shown in figure 2.2.

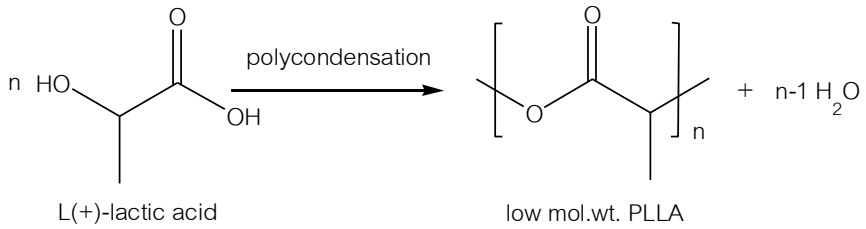

Figure 2.2 Synthesis of low molecular weight of poly (L-lactic acid) by linear polycondensation of L-lactic acid.

The second step is the lactide formation from depolymerization of LPLA prior to obtain LPLA by thermal decomposition as shown in figure 2.3.

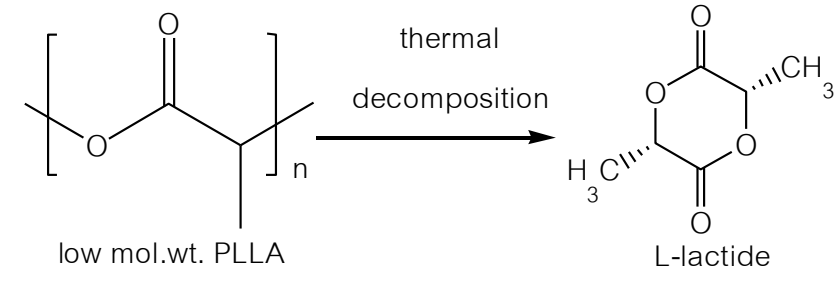

Figure 2.3 Synthesis of lactide by thermal decomposition reaction.

PLA can be obtained by ring opening polymerization of lactide with catalyst as shown in figure 2.4

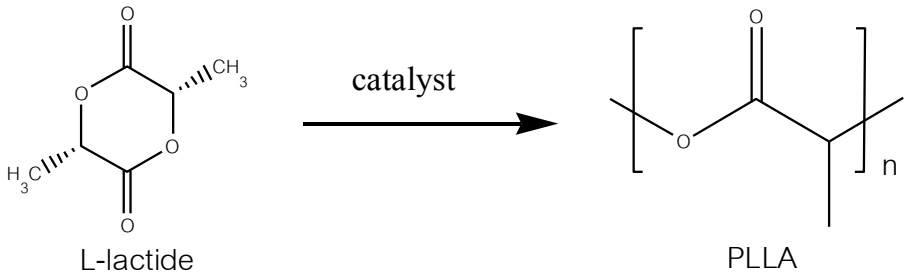

Figure 2.4 Ring opening polymerization of lactide to PLLA

The advantages of the direct poly condensation over the ring opening polymerization such as, easily to produced because it has a less step and also less expensive.

2.2 Polycaprolactone (PCL)

Polycaprolactone (PCL) is a biodegradable polyester with a low melting point of around 60°C and a T_g of about -60°C. PCL is derived from crude oil by chemical synthesis. It can be prepared by ring opening polymerization of ε-caprolactone using a catalyst such as stannous octanoate. Polycaprolactone has good water, oil, solvent and chlorine resistance. PCL is used mainly in thermoplastic polyurethanes, resins for surface coatings, adhesives and synthetic leather and fabrics. It is also used to make stiffeners for shoes and orthopedic splints, and fully biodegradable compostable bags, sutures, and fibers. PCL is degraded by hydrolysis of its ester linkages in physiological conditions (such as in the human body) and has therefore received a great deal of attention for use as an implantable biomaterial. In particular it is especially interesting for the preparation of long term implantable devices, owing to its degradation which is even slower than that of polylactide.

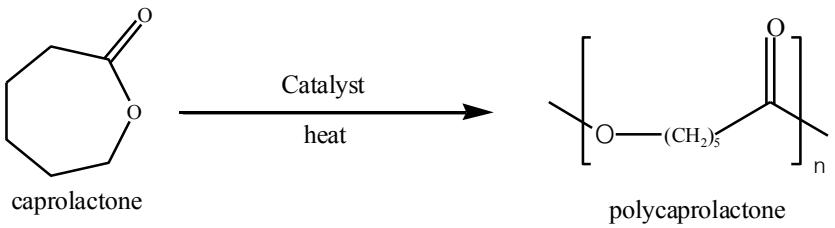

Figure 2.5 Ring opening polymerization of ε-caprolactone to caprolactone.

2.3 Polymer Blend

Blending two polymers is an approach to develop new biomaterials exhibiting combinations of properties that could not be obtained by individual polymers (Santin et al. 1996). Blends made of synthetic and natural polymers can imbibe the wide range of physicochemical properties and processing techniques of synthetic polymers as well as the biocompatibility and biological interactions of natural polymers (Sahoo *et al.* 2010).

Chen *et al.* (2002) prepared and characterized the biodegradable PLA polymeric blends. The effects of the addition of plasticizers or surfactants/compatibilizers were studied. In conclusions, adding PDLLA and surfactant to PLLA via solution-blending may be an effective way to make PLLA tougher and more suitable to use in orthopedic or dental applications. Elongation of PLLA was increased with the addition of PCL, but the strength decreased at the same time.

Wang *et al.* (1998) studied the effect of catalysts/coupling agents on the properties of blened PLA/PCL. Triphenyl phosphite showed the most promising results as a coupling or branching agent. The elongation of the PLA/PCL blends was improved significantly when compared to pure PLA.

In 2010, Sahoo *et al.* also discoverd the blending of chitosan with polycaprolactone. The postulation is that blending chitosan and PCL will give a superior biomaterial where the restrictions of chitosan are complemented by PCL. The

drug release was observed at different pH medium and it was found that the drug release depends upon the pH medium as well as the nature of matrix.

2.4 Wound dressing device

After an injury or surgical procedure, the healing process begins. This consists of three phases and is found in all normal wound healing. These phases are not distinct, but form a continuum of the wound healing process. The inflammatory or substrate phase begins immediately after wounding and lasts approximately 4 days. The initial goal of this phase is hemostasis. This is carried out through smooth muscle contraction and subsequent occlusion of the larger damaged blood vessels. The activation and aggregation of platelets and release of clotting factors at the vessel wall injury site starts the coagulation process that helps to thrombose the smaller damaged vessels (James R. *et al*, 1997).

The dressing will then protect the wound site from further contamination. Maceration of the enclosing skin can be prevented by the absorption of excess wound exudate by the dressing. Reepithelialization and cellular migration take place best in a humid environment, thus desiccation should similarly be avoided. Wound dressing should also be gas permeable-allowing of oxygen to the wound site as well as elimination of carbon dioxide. The incidence of hematoma or abscess formation can be decreased by avoiding wound "dead spaces" through the use of compressive dressings.

To prevent the failure during wound healing process, wound management aids are important to promote the healing process. Wound management is performed by initial cleansing and debridement. After antiseptic procedures are completed, the wound has to be covered with dressing. An ideal wound dressing should provide the optimal environment to meet the treatment objective and protection from further injury. Table 2.1 shows the characteristics of the ideal wound dressing. Generally, the dressing should be capable of speeding up the healing process, preventing infection, and avoiding second injury at the end of healing process. In addition, an antibiotic is

commonly applied to the dressing for reducing inflammation caused by bacterial infection. There are several dressing products commercially available which possess different description and indication. The examples of the adhesives those are currently in clinical use include fibrin sealants, albumin-based compounds, cyanoacrylates, hydrogels, and collagen-based adhesives. Although those dressing devices exhibited the satisfactory performance, the application of an inappropriate dressing may adversely affect wound healing process. Therefore, care must be taken during wound management (Sriputtirat *et al*. 2011).

Table 2.1 Characteristics of the ideal wound dressing

Characteristics	Rationale
Promotes moist wound healing*	Dry wound bed inhibits wound healing
Manages excess exudate	Prevent maceration and further wound breakdown
Provides thermal insulation	Reducing temperature at wound bed reduces fibroblast activity
Impermeable to microorganisms	Prevent exit and entry of organisms
Causes minimal trauma on removal	Prevents damage and reduces pain
Cost effective	Makes best use of available resources
Available in hospital and community	Accessible to all carers

Remark: in certain circumstances, moist wound healing may not be the treatment objective, e.g. black heels

2.5 Homeostasis

When large vessels are cut or torn, the rapid flow of escaping blood tends to remove fibrin clots before they can clog the vessel and adhere to the adjacent damaged tissue. There is a requirement for material able to arrest such major hemorrhage. In many cases, blood adhering to damage tissue is clotted and slowly transformed into a scab that serves as a skin substitute. The scab retains body fluids while sealing out bacteria and other environmental hazards. Wound healing normally takes place under cover of the protective scab, which prevents drying of underlying cells and undesirable inflammatory reactions which limit normal healing. Such healing requires closure of any void with fibroblasts and the migration of epidermal cells over fibroblasts and fibroblastic collagen products under physiological conditions. There is a need for methods able to rapidly provide the protective functions normally provided by epithelial cells and to foster re-epithilialization for re-establishment of such functions. An artificial scab is expected to provide such immediate protection. Polymers in forms of aqueous gel, mucilage, glue, cream, granule, and particle, such as polyvinyl alcohol, polyvinyl acetate, polyphosphate, poly-4-hydroxybutyrate, and chitosan, are now commercially available to prevent trauma bleeding by providing the template for scab formation. Polyvinyl alcohol and polyvinyl acetate in form of gel helped cover and adhere to the open wound, denuded tissue, or burned skin so that bleeding or fluid loss was reduced or stopped. It was reported that polyphosphate modulated blood coagulation and fibrinolysis; thus, reduced bleeding. Carboxymethyl chitosan bandage has been proved to treat burned skin and prevent infection and skin inflammatory. Chitosan granule containing poly-4-hydroxybutyrate can be used to treat intracavity bleeding (Ruiz et al. 2004, Smith et al. 2006, Wynd *et al.* 1944, and Ahuja *et al.* 2007). Not only immediate clotting, biocompatibility and antimicrobial activity are also the requirements in treatment of hemorrhage.

Chitosan's properties allow it to rapidly clot blood, and have recently gained approval in the US for use in bandages and other hemostatic agents. Chitosan purified from shrimp shells is used in a granular hemostatic product, Celox, made by Medtrade Biopolymers Inc. (Crewe, England) and in the chitosan dressings made by HemCon

Medical Technologies Inc. (OR, USA). Celox has been shown in testing by the US Marines to quickly stop bleeding and result in 100% survival of otherwise lethal arterial wounds and to reduce blood loss. The HemCon product reduces blood loss in comparison to gauze dressings and increases patient survival. In addition, chitosan is hypoallergenic, and has natural antibacterial properties, further supporting its use in bandages (Ahuja *et al.* 2007).

In clotting process, the role of biomaterials is to provide the supported network where platelets aggregate and fibrin-based clot forms on demand by body response. In case of severe bleeding, use of active ingredient to speed up clotting process and further reduce clotting time can help prevent trauma hemorrhage.

2.6 Blood clotting

Control of bleeding is complicated by many factors because clotting is a complex process involving multiple interdependent interactions among platelets, endothelial cells, white cells, and plasma proteins. Although blood clotting process is not yet understood in all details, it can be simply defined in 3 steps. First, the prothrombin-heparin complex reacts with the cephalin freed by the disintegrating platelets to form prothrombin and cephalo-heparin. Second, prothrombin forms thrombin by a reaction which involves free calcium ions. Last, thrombin and fibrinogen are reacted and the fibrin-based clot is formed (Gentry *et al.* 2004, Urata *et al.* 2003, and Mershon *et al.* 2007).

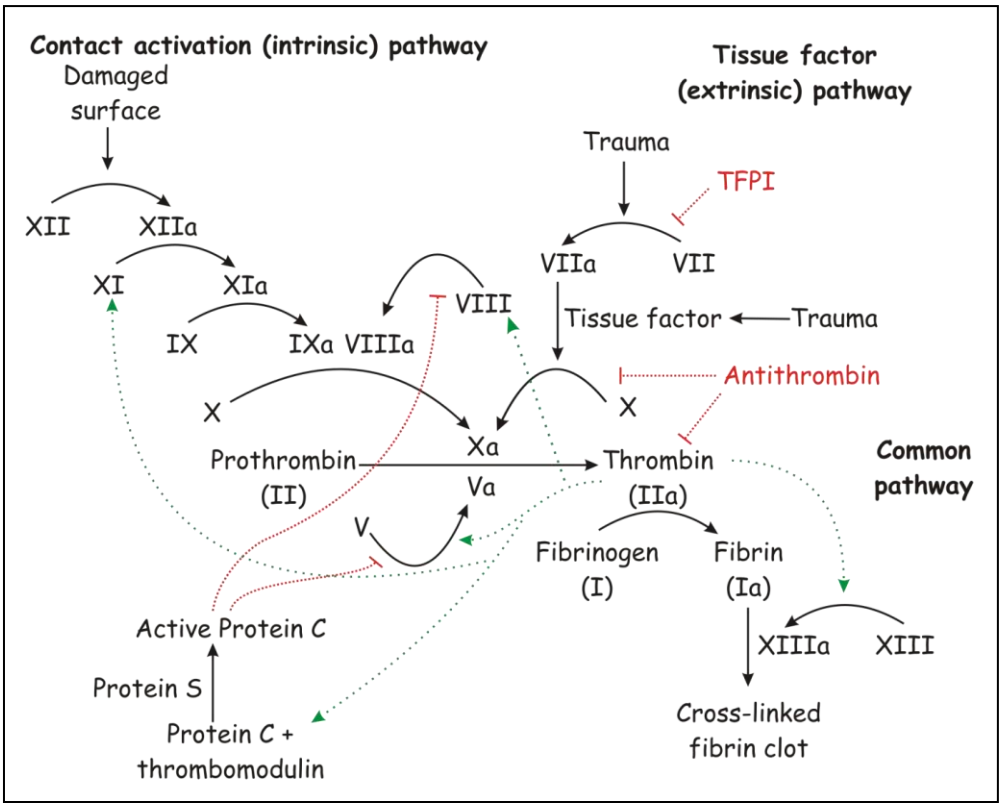

Figure 2.6 Blood coagulation process (http://en.wikipedia.org/wiki/Coagulation)

2.6.1 PT,aPTT

Definition of prothrombin time (PT) and partial thromboplastin time (aPTT)

The prothrombin time (PT) and its derived measures of prothrombin ratio (PR) and international normalized ratio (INR) are measures of the *extrinsic pathway* of coagulation. They are used to determine the clotting tendency of blood, in the measure of warfarin dosage, liver damage, and vitamin K status. PT measures factors I, II, V, VII, and X. It is used in conjunction with the activated partial thromboplastin time (aPTT) which measures the *intrinsic pathway*.

The partial thromboplastin time (PTT) or activated partial thromboplastin time (aPTT or APTT) is a performance indicator measuring the efficacy of both the "intrinsic" (now referred to as the contact activation pathway) and the common coagulation pathways. Apart from detecting abnormalities in blood clotting,[1] it is

also used to monitor the treatment effects with heparin, a major anticoagulant. It is used in conjunction with the prothrombin time (PT) which measures the *extrinsic pathway*. Kaolin cephalin clotting time (KccT) is a historic name for the activated partial thromboplastin time.

2.7 Antibacterial activity

Bacteria are a large biology of single-cell prokaryotes microorganism, quite different from the eukaryotes which include the fungi, plants and animals. Bacteria have a single circular DNA chromosome that is found within the cytoplasm. They are often harmful as the causes of human and animal illness and they are found in the environment as either individual cell or aggregated together as clusters, and their intracellular structure is far simpler than eukaryotes.

2.7.1 Cell Morphology

Bacteria display a wide diversity of cell morphology. Typical shapes of bacteria include:

- coccus (spherical)
- bacillus (rod-like)
- spirillum (spiral)
- filamentous

In fact, however, bacteria come in a wide variety of shapes and sizes, called the morphology of the organism. The most common shapes are rod-like, called the bacillus form, or spherical, called the coccus form. The rod forms vary considerable from short rods that almost look like cocci, to very long filaments thousands of microns in length. Bacteria also form spirals and corkscrews, ovals, commas, and elaborately branched structures. The cocci often take on multi-cell forms; as two cocci joined together, as chains of coci or as tetrads.

2.7.2 The Bacterial Cell Wall

As in other organisms, the bacterial cell wall provides structural integrity to the cell. In prokaryotes, the primary function of the cell wall is to protect the cell from internal turgor pressure caused by much higher concentrations of proteins and other molecules inside the cell compared to its external environment. The bacterial cell wall differs from that of all other organisms by the presence of peptidoglycan (poly-*N*acetylglucosamine and *N*-acetylmuramic acid), which is located immediately outside of the cytoplasmic membrane. Peptidoglycan is responsible for the rigidity of the bacterial cell wall and for the determination of cell shape. It is relatively porous and is not considered to be a permeability barrier for small substrates. While all bacterial cell walls (with a few exception e.g. intracellular parasites such as *Mycoplasma*) contain peptidoglycan, not all cell wall have the same overall structure. There are two main type of bacterial cell wall, Gram positive and Gram negative, which are differentiated by their Gram staining characteristics. As briefly noted before, fundamental differences in ultrastructure of the cell wall are responsible for the reaction (+ or -) of bacteria towards the Gram stain. In both types of cell, the cytoplasmic membrane is surrounded and supported by a cell wall, which provides strength, rigidity and shape. Schematic cross sections of these structures are given below.

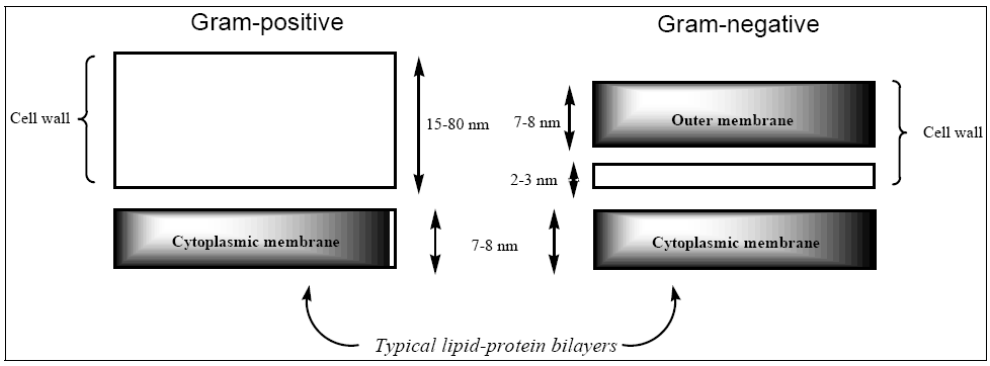

Figure 2.7 Structures of cell wall of gram-positive and gram-negative bacteria.

2.7.2.1 The Gram Positive Cell Wall

Gram-positive bacteria differ from gram-negative bacteria in the structure of their cell walls. The cell walls of gram-positive bacteria are made up of twenty times as much murein or peptidoglycan than gram-negative bacteria. These complex polymers of sugars and amino acids cross-link and layer the cell wall. The thick outer matrix of peptidoglycan, teichoic acid, polysaccharides, and other proteins serve a number of purposes, including membrane transport regulation, cell expansion, and shape formation. Almost all bacteria can be classified as gram-positive or gram-negative. The classification relies on the positive or negative results from Gram's staining method, which uses complex purple dye and iodine. Because gram-positive bacteria have more layers of peptidoglycan in their cell walls than gram-negative, they can retain the dye. (See Figure 2.8)

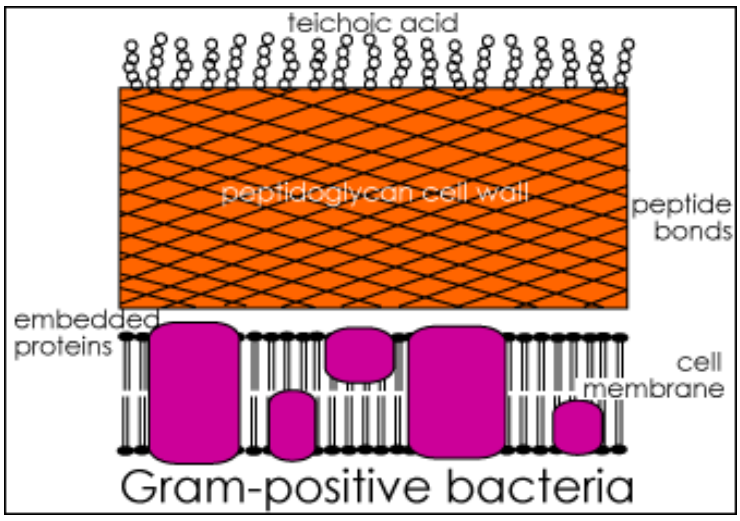

Figure 2.8 Gram-positive bacteria cell wall
(http://www.hhmi.org/biointeractive/Antibiotics_Attack/bb_1.html)

2.7.2.2 The Gram Negative Cell Wall

Both gram-positive and gram-negative bacteria have a cell wall made up of peptidoglycan and a phospholipid bilayer with membrane-spanning proteins. However, gram-negative bacteria have a unique outer membrane, a thinner layer of

peptidoglycan, and a periplasmic space between the cell wall and the membrane. In the outer membrane, gram-negative bacteria have lipopolysaccharides (LPS), porin channels, and murein lipoprotein all of which gram-positive bacteria lack. As opposed to gram-positive cells, gram-negative cells are resistant to lysozyme and penicillin attack. The gram-negative outer membrane which contains LPS, an endotoxin, blocks antibiotics, dyes, and detergents protecting the sensitive inner membrane and cell wall. LPS is significant in membrane transport of gram-negative bacteria. LPS, which includes O-antigen, a core polysaccharide and a Lipid A, coats the cell surface and works to exclude large hydrophobic compounds such as bile salts and antibiotics from invading the cell. O-antigen are long hydrophilic carbohydrate chains (up to 50 sugars long) that extend out from the outer membrane while Lipid A (and fatty acids) anchors the LPS to the outer membrane. (See Figure 2.9)

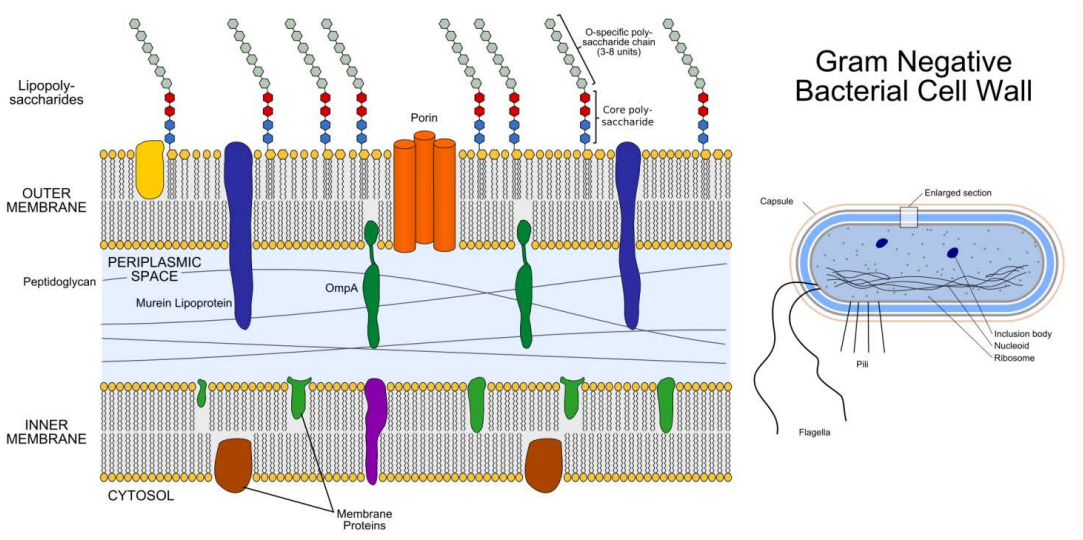

Figure 2.9 Gram-negative bacteria cell wall
(http://en.wikipedia.org/wiki/Bacterial_outer_membrane)

2.7.3 Bacterial Growth Curve

Bacterial growth over time can be graphed as cell number versus time. This is called a growth curve. The cell number is plotted as the long of the cell number, since

it is an exponential function. Regardless of the generation time, in a growing culture the plot of a log of cell number versus time gives a characteristic curve. This curve typically has four distinct phases: lag phase, exponential (log) phase, stationary phase, and death phase.

In cells that have been freshly inoculated into a new growth medium, the **lag phase** is the first phase observed. It is characterized by no increase in a cell number; however, the cell are actively metabolizing, in preparation foe cell division. Depending on the growth medium, the lag phase maybe short or very long. For example, if a culture in a rich growth medium that supplies most of cells' requirements is inoculated into a poor medium that requires the cells to make most of their own amino acids and vitamins, the lag phase will be very long. The cell must activate metabolic pathways for amino acid and vitamin synthesis and must make enough of these nutrients to begin active growth. In contrast, cells that are simply diluted from one medium to a fresh tube of the same medium may show virtually no lag phase, since they need not change their metabolism.

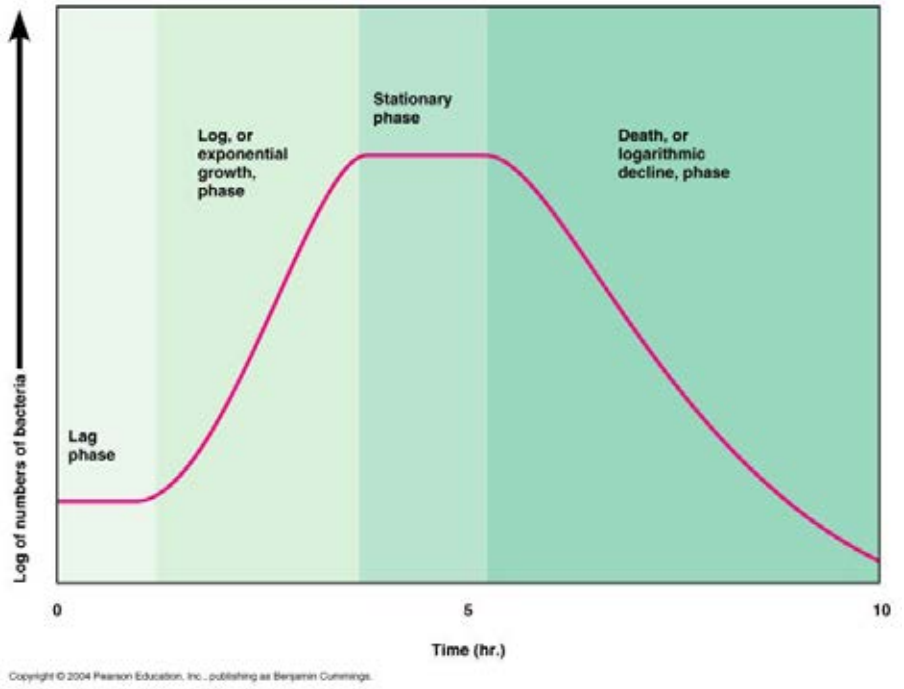

Figure 2.10 Bacterial growth curve showing the four phases of growth versus time.
(Zwietering *et al*. (1990))

Once cells are actively metabolizing they begin DNA replication and shortly after that the cells divide. This begins the second phase of growth call **exponential or log phase** of growth. This is the period in which the cells grow most rapidly, doubling at a fairly constant rate. The time it takes the culture to double is called the generation time can be easily obtained from the exponential phase of a growth curve. The log of the cell number versus time will yield a straight line when the cells are exponential growth. The generation time can be read directly from the graph using two points on the straight line that represent a two-fold increase in the cell number.

The generation time depends on several factors: the organism itself, the growth medium, and the temperature are all important factors in determining the generation time. Under constant conditions, the generation time for any organism is quite reproducible, but differs greatly among different bacteria. The fastest growing bacteria have generation times of 15-20 min under optimum growth conditions. Many bacteria, however, have generation times of hours or even days.

The third phase in the growth of bacteria is **stationary phase,** when metabolism slows and the cells cease rapid cell division. They may divide slowly for a time, but soon stop dividing completely. They are still alive and maintain a slow metabolic activity. The factors that cause cell density. Among the changes that slow growth are depletion of nutrients and accumulation of waste products. If cells in stationary phase are diluted into fresh medium the quickly resume exponential growth.

The final phase of the growth cycle is **death phase**. In this phase the cells quickly lose the ability to divide even if they are placed in fresh medium. Like the phase of rapid growth, the death phase is also exponential; therefore, cells die quickly and within hours a culture may have no living cells. The death phase, and in fact all the phases, can be slowed by lowering the temperature. Hence, in order to maintain maximum cell viability it is best to grow bacterial cultures only to early stationary phase and then chill the culture. Leaving a culture at the optimum temperature for growth for a long period of time simply accelerates the death of the culture. Most

dead bacterial cells look identical to live cells, so the normal appearance of a liquid culture or of colonies on a plate is no indication that the cell alive.

2.8 Chitosan

Chitosan, a natural, nontoxic, edible and biodegradable polymer, derived by deacetylation of chitin (poly-*N*-acetyl-glucosamine), is the second most abundant biopolymer in nature after cellulose. The chemical structure of chitosan is shown in Figure 2.10. In general, chitin is present in the exoskeleton of arthropods such as insects, crabs, shrimps and certain fungal cell walls. At present, several interesting biological properties have been reported for chitosan, such as wound healing, immunological activity and antibacterial effects.

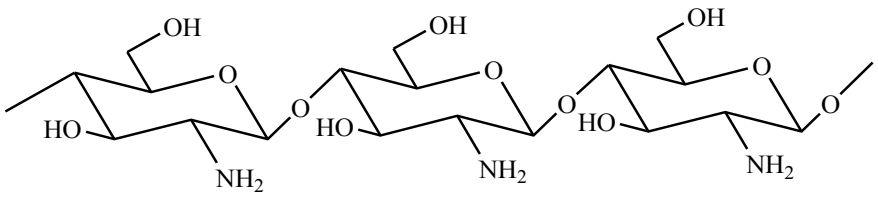

Figure 2.11 The chemical structure of chitosan.

Aoyagi *et al*. (2007) prepared the novel wound dressings composed of chitosan film and minocycline hydrochloride. Chitosan also promotes wound and burn healing properties, enhances the functions of inflammatory cells such as polymorphonuclear leukocytes, macrophages, fibroblasts and it is beneficial for the large open wounds of animals.

Ong *et al.* (2008) refined the chitosan wound dressing by incorporating a procoagulant (polyphosphate) and an antimicrobial (silver). The optimal chitosan-polyphosphate formulation (ChiPP) accelerated blood clotting, increased platelet adhesion, generated thrombin faster, and absorbed more blood than chitosan. Silver-loaded ChiPP exhibited significantly greater bactericidal activity than ChiPP.

2.8.1 Hemostatic activity

In the wound healing process hemostasis is the first step. Platelets, the protagonists in blood coagulation, release some cytokines that enhance the healing. The mechanism by which chitin and chitosan act as hemostatics has been investigated, and many authors have concluded that chitosan influences platelets.

Okamoto *et al.* (2003) studied the effects of chitin and chitosan on blood coagulation and platelet aggregation. Whole blood was mixed with chitin and chitosan suspensions, and then the blood coagulation time (BCT) was measured using the modified Lee-White method. Chitin and chitosan reduced BCT in a dose-dependent manner. Platelet rich plasma (PRP) was mixed with chitin and chitosan suspensions, and then platelet aggregation (PA) was measured using a Dual aggregometer. The PA level induced by chitin was the strongest in all samples including chitosan, cellulose, and latex. When the washed platelet was used, the PA level induced by chitin was similar to that of chitosan, while the rate of coagulation was lower than that of PRP. Chitin and chitosan enhanced the release of the platelet.

Ahuja *et al.* (2007) prepared a chitosan embedded in a polymer mesh material of poly-4-hydroxy butyrate, or has interspersed with it a polymer mesh material of poly-4-hydroxy butyrate. The granule or particle can be used to treat intracavity bleeding.

2.9 Doxycycline as an antibacterial drug

Doxycycline is a member of the tetracycline antibiotics group and is commonly used to treat a variety of infections. Doxycycline works by interrupting the production of proteins by bacteria. It is effective against a wide variety of bacteria, such as *Hemophilus influenzae*, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Neisseria gonorrhoea*, and many others.

Doxycycline is used in the treatment of infections of the skin, bone, stomach, respiratory tract, sinus, ear, and urinary tract. Lyme disease and certain sexually transmitted diseases (gonorrhea and chlamydia) can also be treated with Doxycycline.

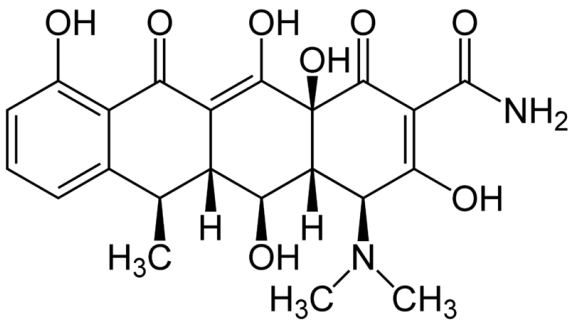

Figure 2.12 Structure of Doxycycline

Toxicological Information

Handling of the formulated product is not expected to cause any adverse affects. The following data pertains particularly to the active and any hazardous ingredients in the formulation, rather than this specific formulation.

Acute Health Effects

Ingestion

Eye Eye contact is not a great risk as this product is a tablet. The data available suggests that eye contact may result in a transient irritation and associated redness and pain.

Skin Skin contact is not a great risk as this product is a tablet. The data available suggests that skin contact may result in mild discomfort. Sensitisation may occur and results in allergic dermatitis, rashes, itchiness, hives and swelling of the extremities.

Inhalation Inhalation is not a great risk as this product is a tablet. Inhalation of the dust may be discomforting to the upper respiratory tract. Pre-existing respiratory disease may be exacerbated by inhalation of the dusts.

Chronic Health Effects

Ingestion Chronic ingestion is unlikely however can cause sore throat, hoarseness, black hairy tongue, bulky loose stools, fat in the faeces, inflammation of the oral cavity, difficulty swallowing, damage to the anogenital area and oesophageal ulcers. Vitamin K function and clotting are affected.

Eye The data available suggests that repeated or prolonged exposure may lead to conjunctivitis. Deposits in the eyes (from systemic use) can cause an abnormal pigmentation of the conjunctiva. Hypersensitivity reactions include burning of the eyes and conjunctivitis.

Skin The data available suggests that repeated or prolonged contact may be discomforting to the skin. Hypersensitivities can produce red rashes, dermatitis with sloughing, hives and swollen cracked lips. Loosening and pigmentation of the nails can also occur.

Inhalation The data available suggests that repeated or prolonged exposure can induce hypersensitivities with signs as described above.

2.9.1 Action of antibacterial drug

Doxycycline elicits its antimicrobial activity by preventing the addition of amino acids to growing peptide chains in bacteria (Lynn, 1996). Apart from antimicrobial properties doxycycline also acts as an inhibitor of matrix metalloproteinases (MMPs) (Nordstrom *et al*., 1998), which is attributed to its metal-binding property. Evidence from animal studies shows that treatment with doxycycline improves healing parameters, like increasing the tensile strength of rat intestinal anastomoses after surgery and reducing the incidence of ulceration in alkali-injured rabbit eyes (Siemonsma *et al*., 2003).

2.10 Oleanolic acid

Oleanolic acid (3/3-hydroxy-olea-12-en-2%oic acid) and its isomer, is triterpenoid compounds which exist widely in natural plants in the form of free acid or aglycones for triterpenoid saponins (Price *et al*., 1987; Mahato *et al*., 1988; Wang and Jiang, 1992). Saponins can be chemically categorized as comprising an aglycone linked to one or more sugar chains. There are two groups of saponins, one contains a steroidal aglycone, and the other contains a triterpenoid aglycone (Price *et al*., 1987). Squalene is considered as the common precursor for biosynthesis of both steroid and triterpenoid systems (Price et al., 1987). Like steroids, triterpenoids have many biological effects, and interest in triterpenoids is growing (Price et al., 1987; Mahato *et al*., 1988). In this review, discussion will be focused on pharmacology of the two triterpenoids, oleanolic acid and its isomer, ursolic acid, largely because they share many common pharmacological properties. Other triterpenoids may have similar properties but in general they have not been studied in as much detail.

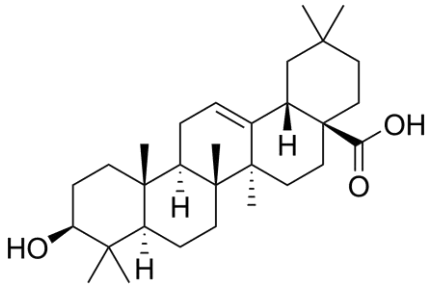

Figure 2.13 Structure of oleanolic acid

Lee *et al.* (2007) investigated the effects of oleanolic acid on the aggregation of rabbit platelets and a mechanism of it. They found that, oleanolic acid at concentrations of 25, 50, 100 and 200 μM induced the aggregation of washed rabbit platelets.

2.11 Monosodium glutamate

Monosodium glutamate (MSG) is one of several forms of glutamic acid found in foods, in large part because glutamic acid, being an amino acid, is pervasive in nature. Glutamic acid and its salts can also be present in a wide variety of other additives, including hydrolyzed vegetable proteins, autolyzed yeast, hydrolyzed yeast,

yeast extract, soy extracts, and protein isolate, which must be labeled with these common and usual names.

Production and chemical properties

Since the first time that MSG was released into the market, it has been produced by three methods: (1) hydrolysis of vegetable proteins with hydrochloric acid to disrupt peptide bonds, (2) direct chemical synthesis with acrylonitrile, and (3) bacterial fermentation; the current method.

Currently, most of the world production of MSG is by bacterial fermentation in a process similar to wine, vinegar, yogurt and even chocolate. Sodium is added later through the step of neutralization. During fermentation, selected bacteria (coryneform bacteria) cultured with ammonia and carbohydrates from sugar beets, sugar cane, tapioca or molasses, excrete amino acids into the culture broth from where L-glutamate is isolated. Nowadays, the conversion yield and production rate from sugars to glutamate continues to improve in the industrial production of MSG, which allows for keeping up with demand. The final product after filtration, concentration, acidification and crystallization is pure glutamate, sodium and water. It appears as a white and odorless crystalline powder that in solution dissociates into glutamate and sodium. It is freely soluble in water, but not hygroscopic and practically insoluble in common organic solvents such as ether. In general, MSG is stable under the conditions of regular food processing. During cooking, MSG does not decompose, but like other amino acids, browning or Maillard reactions will occur in the presence of sugars at very high temperatures.

Safety information

The Oral lethal dose to 50% of subjects (LD50) is between 15 to 18 g/kg body weight in rat and mice respectively, 5 times greater than the LD50 of Salt (3 g/kg in rats). Therefore, the intake of MSG as a food additive and the natural level of glutamic acid in foods do not represent a toxicological concern in humans.

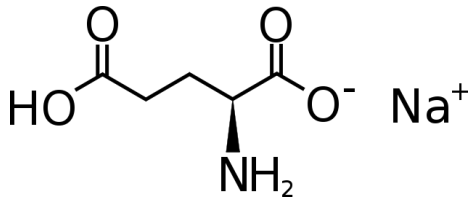

Figure 2.14 Structure of monosodium glutamate

In 2008 Thakkum *et al.* studied the effect of monosodium glutamate (MSG) on blood clotting in rat (*Rattus norvegieus*) and reported that MSG could stop the bleeding and fibrin formation could be observed significantly faster. The results also showed that the treated with MSG for the cessation of bleeding showed thicker fibrin formation at the wound tissue of rat tails than untreated the MSG.

Sodium (Na) molecule is affects on protein clotting factor is Factor IXa (FIXa) (Schmidt *et al*, 2005) by working with FVIIIa which is stimulate the process in the mechanism of blood clotting. The molecule Glutamate is affects to accelerate vitamin K from reducing to oxidizing form, working form and ready to stimulate the necessary Protein Clotting Factor including Factor II (Prothrombin), Factor VII, Factor IX. And Factor X and also stimulating other factor in the blood clotting mechanism (Bowen, 1999; Higdon, 2004; Tollefsen, 2006).

CHAPTER III

EXPERIMENTAL

3.1 Materials

All chemicals were obtained from various suppliers as shown in Table 3.1 and they were used as received.

Table 3.1 Sources of chemicals

Number	Chemical	Company	Grade
1	L-lactic acid (LA) (88% aqueous solution)	Archer Daniels Midland	Commercial
2	Chitosan (CS) (DD = 94.6%, MW 600,000-1,000,000)	Taming Enterprises Co., Ltd.,	Commercial
3	Nitrogen gas	Thai Industrial Gas	High purity 99.99%
4	Methanol (MeOH)	Fluka	Commercial
5	Sodium hydroxide (NaOH)	Carlo ERBA	AR grade
6	Chloroform-d	Aldrich	AR grade
7	Glycerol (Gly)	BDH Laboratory Supplier	AR grade
8	Polyethylene glycol (PEG)	Aldrich	AR grade
9	Polycaprolactone (PCL) (M_w =60,000)	Aldrich	AR grade
10	Oleanolic acid	Sigma	AR grade
11	Tetrahydrofuran (THF)	Aldrich	AR grade
12	Hemostat Thromboplastin-SI	Human	AR grade

Number	Chemical	Company	Grade
13	Hemostat aPTT-EL	Human	AR grade
14	Triplicate soy broth (TSB)	Difco	AR grade
15	Glutaraldehyde	Aldrich	AR grade
16	Dichloromethane	Aldrich	AR grade
17	Doxycycline hyclate	Sigma	AR grade

3.2 Equipments and instrument

All apparatuses used in this experiment were listed in Table 3.2.

Table 3.2 Apparatuses used in the experiment

Number	Instrument	Model	Company/Country
1	Nuclear Magnetic Resonance Spectrometer (NMR)	Varian mercury-400 spectrometer Waters 150-CV Degas: ERC-3415α	Varian, USA
2	Gel Permeation Chromatograph (GPC)	*Column*: Waters Styragel HR columns (HR 1, 3, and 4), PL-gel 10 μm *Pump*: Waters 600 Controller *Refractive Index Detector*: Waters 2414	Waters, USA
3	Differential Scanning Calorimeter (DSC)	NETZSCH DSC204F1 Phoenix	Phoenix, USA
4	Attenuated Total Reflection Infrared (ATR-IR) spectroscopy	NICOLET 6700 FT-IR spectrometer with CONTINUμM IR	USA

		Microscope	
Number	**Instrument**	**Model**	**Company/Country**
5	Universal Testing Machine (UTM)	5583 Serial#11202H	USA
6	Vacuum Drying Oven	DP 41	Yamato Scientific, Japan
7	Centrifuge	KR-20000T	Shimadzu, Japan
8	Hot Air Oven	FG 32R	Japan
9	Shaker	INFORS AG CH-4103 Bottmingen	Switzerland
10	Homogenizer	NISSEI AM-11	Japan
11	UV-vis spectrophotometer	UV-160	Shimadzu, Japan

3.3 Experiment

3.3.1 Preparation of CHI/PLA/PCL blend film

Poly(L-lactic acid) was prepared by direct polycondensation of L-lactic acid. L-lactic acid solution was heated at 140°C under N_2 atmosphere for 2 h. Water was removed from the reaction mixture by applied high vacuum pressure for 30 min. After that the reaction mixture was heated to 160°C until no condensate was present. Low molecular weight poly(L-lactic acid), so called LPLA, was heated to 200°C under vacuum with the presence of 3 different catalysts including $Sn(Oct)_2$, $Co(OAc)_2$, and $Sb(OAc)_2$. The selected solvent system was diphenyl ether. The reaction time was varied at 24, 48, 72, and 96 h. The resulting PLA crystal was collected by dissolving the reaction mixture in $CHCl_3$, and precipitating by methanol. The crystal was then dried under vacuum at 50°C for 24 h.

Firstly, PLA/PCL blend was prepared by dissolving PLA and PCL in dichloromethane at the fixed weight ratio of 1:1 (50%:50%). While chitosan solution

(2% wt) was prepared by dissolving chitosan flake into lactic acid solution (1% vol). CHI/PLA/PCL blend at 3 different weight ratios of CHI to PLA/PCL blend (30:70, 40:60, and 60:40) was prepared by the modified spontaneous emulsification-solvent diffusion method (See Table 3.3 for more details). PLA/PCL blend was gently dropped into 2% chitosan solution with the presence of glycerine or polyethylene glycol (PEG) at 0.5% wt as an emulsifier. The mixture was stirred at 12,000 rpm. The solution was poured onto the petridish and the solvent was allowed to evaporate at ambient condition. The film was then dried at 30°C for 72 h and kept under vacuum until use.

To investigate if the obtained CHI/PLA/PCL blend film represent all crucial properties of each biopolymer used, the blend film was characterized by an attenuated total reflection infrared (ATR-IR) spectroscopy to determine the actual compositions in the film and a differential scanning calorimetry (DSC) technique to observe the deviation in a glass transition temperature (Tg) of the polymer blend from those of the homopolymer.

Table 3.3 Blend film compositions with the fixed weight ratio of the PLA/PCL at 1:1

Composition ratio (CHI:PLA/PCL) (% wt)	**Total film weight (g)**	**CHI (g)**	**PLA/PCL (g)**
30:70	0.5	0.15	0.35
	1	0.30	0.70
40:60	0.5	0.20	0.30
	1	0.40	0.60
60:40	0.5	0.30	0.20
	1	0.60	0.40

After obtaining the optimal ratio of CHI to PLA/PCL that gave the desired chemical and thermal properties as determined by the techniques mentioned above,

the mechanical and controlled release properties of the film was improved by varying the blend ratios of PLA to PCL while keeping the constant CHI to PLA/PCL weight ratio at 60:40 (Table 3.4). The preparation method was similar to those mentioned earlier.

The prepared CHI/PLA/PCL blend film was investigated for the improvement in the mechanical properties. Those properties include tensile strength (TA), elongation percentage at break (%E), and bioadhesive strength (T-peel test). The water vapor transmission rate (WVTR) and the swelling degree were also investigated.

Table 3.4 Blend film compositions

(CHI:PLA:PCL) (% wt)	Total weight (g)	CHI (g)	PLA (g)	PCL (g)
60:4:36	1.0	0.60	0.04	0.36
60:12:28	1.0	0.60	0.12	0.28
60:20:20	1.0	0.60	0.20	0.20
60:28:12	1.0	0.60	0.28	0.12
60:36:4	1.0	0.60	0.36	0.04

Controlled release property of the prepared film was observed by loading doxycycline as a model drug into the chitosan solution before blending with PLA/PCL blend. The doxycycline loaded film was casted according to the method previously described. The obtained film was cut into a square shape (30 mm × 30 mm). The film thickness was measured by veneer caliper and found to be 0.1 mm. The square shape film was then immersed into the PBS buffer (pH 7.4). The flask was then incubated at 37°C and 110 rpm. 3 mL sample was taken from the flask and 3 mL fresh PBS was replaced to maintain the constant volume during the test. The sample was filtrated and diluted before measuring UV absorption at 274 nm (UV-Vis

spectrophotometer). The amount of doxycycline released was calculated from a calibration curve (the prepared concentration of doxycycline versus the reading of UV absorption at 274 nm). A cumulative correction was also made for the previously removed sample to determine the total amount of drug release. All experiments were performed 3 times.

The amount of doxycycline released from the film was investigated over the incubation time (2 h) and compared with the initial loaded amount onto the film. Doxycycline released into PBS buffer was determined as described above. The total amount of doxycycline released from the film was compared with the initial amount loaded on the film. The release rate and percentage of doxycycline from the film were then calculated by the following equation.

$$\%\ Doxycycline\ release = \frac{amount\ of\ Doxycycline\ released}{amount\ of\ Doxycycline\ loaded} \times 100$$

3.3.2 In vitro controlled release of active ingredients

3.3.2.1 In vitro blood clotting

Monosodium glutamate (MSG) and oleanolic acid were studied for their application as the active ingredients to accelerate blood clotting process. Model MSG and oleanolic acid solution were tested at various concentrations with human blood plasma to investigate their concentration effect on the Prothrombin time (PT) and the activated partial thromboplastin time (aPTT). Human blood plasma was prepared from the whole human blood supplied by the Thai Red Cross Society.

The method described by Brown (1988) was used for the determination of PT. Plasma was obtained by centrifugation of citrated blood for 15 min at 1,500g. Thromboplastin-calcium reagent was reconstituted with distilled water. The mixture was prewarmed in a water bath at 37°C for at least 10 min before the test. 100 μL plasma was transferred into the test tube and incubated in the water bath for 180 s. For the control, 100 μL prewarmed PBS and 200 μL prewarmed thromboplastin-calcium

reagent were rapidly added into the plasma in the test tube. The test tube was then gently tilted until the clot was formed. The clotting time (PT) was defined as the time when the clot was formed. For the sample, 100 µL prewarmed sample solution in PBS was mixed with the plasma. After that, the mixture was added into thromboplastin-calcium reagent in the test tube.

APTT was also determined according to the method described by Brown (1988). For the control, Alexin (partial thromboplastin with activator) and 0.02 M $CaCl_2$ were prewarmed at 37°C. 50 µL plasma was transferred into the test tube. After 180 s incubation, 50 µL Alexin was added into the reaction mixture. The mixture was incubated for another 180 s prior to the addition of 50 µL PBS. 50 µL 0.02 $CaCl_2$ was added into the mixture. The mixture was mixed thoroughly. The test tube was maintained in the water bath. The test tube was gently tilted every 5 s. At the end of 20 s, the test tube was removed from the water bath, and further gently tilted until the clot was formed. The clotting time was defined as APTT. For the sample, 50 µL PBS was replaced with 50 µL sample.

3.3.2.2 Blood clotting test on MSG loaded CHI/PLA/PCL film

Compared with oleanolic acid, MSG was found to be active in accelerating in vitro blood clotting (3.3.2.1). Later, only MSG was selected for preparing the blend film. The similar protocol to the preparation of doxycycline loaded CHI/PLC/PCL blend film was used in preparing MSG loaded CHI/PLA/PCL blend film. In *vitro* MSG release was investigated under the similar condition to that with the doxycycline loaded film. MSG released into the buffer solution was then used to test for its effect on human blood clotting by observing the change in PT and aPTT.

Blood and platelet adhesion onto the film surface was observed from scanning electron micrographs. Each MSG loaded film was immersed in whole citrated human blood and platelet rich plasma at 37°C. After 1 h, the film sample was carefully rinsed by the saline solution to wash off the free blood cells. The adhered blood cells on the film surface were fixed by immersing the film into the saline solution containing

glutaraldehyde (2.5% wt) at room temperature for 30 min. Then, the film sample was carefully rinsed three times by the saline solution. Prior to freeze drying step, the film sample was rinsed by distilled water to eliminate salts deposited onto the film surface. Later, the film sample was cut into the desired size and vacuum coated with carbon and gold before observation under the SEM.

3.3.3 Antibacterial activity test

3.3.3.1 Growth kinetic monitoring

Triplicate soy broth (TSB) was used as a growth medium for the antibacterial activity assays. *Staphylococcus aureus* and *Escherichia coli* were used as the bacterial representatives, gram positive and gram negative, respectively. The negative and positive controls were a broth solution with bacterial suspension and a broth solution with doxycycline/MSG loaded CHI/PLA/PCL, respectively. All glasswares used for the tests were sterilized in an autoclave at 121°C for 15 min prior to use. Wound dressing films were sterilized by exposing to UV radiation for 30 min prior to the tests.

Bacterial suspension was prepared from the fresh slant of *S. aureus* and *E. coli*. 0.3 mL bacterial suspension (both *S. aureus* and *E. coli*) in distilled water was transferred into a flask containing 50 mL TSB. Neutralized CHI, CHI/PLA/PCL blend film, PLA film, boots stop bleeding fast dressing (contain micro-dispersed oxidised cellulose), and doxycycline/MSG loaded CHI/PLA/PCL blend film were tested for their antibacterial activity. Each film (2×2 cm^2 in size) was added into in the bacterial suspension and the bacterial culture was shaken at 250 rpm and 37^{o}C. Three experimental replicates were conducted for each film sample. The bacterial suspension was sampled to measure the optical density at 600 nm (OD600) by UV-Vis spectroscopy every 3 h. The cell dry weight was also determined from the sample taken every 3 h. The total bacterial cell number present in the culture broth (TSB) was determined from the obtained results of OD600 reading and the cell dry weight data.

Viable bacterial cell number present in the culture broth at various times was also determined using the plate count technique to investigate the antibacterial activity of the loaded ingredients onto the film or the properties of the film itself. From the bacterial suspension sample that was taken every 3 h, each was diluted 10^{12} times. 100 µL diluted sample was spreaded onto the triplicate soy agar (TSA) plate. After incubating at 37°C for 24 h, the numbers of bacterial colonies growing on the plate were counted. The result after multiplying by the dilution factor (10^{12}) and dividing by the volume of the diluted suspension spreaded onto the plate represented the mean colony forming units per volume (CFU/mL). The antibacterial ratio was calculated using the following equation.

$$Antibacterial\ ratio = \frac{Number\ of\ original\ cell - Number\ of\ viable\ cell}{Number\ of\ original\ cell} \times 100$$

3.3.3.2 Determination of bacterial adhesion on film surface

At every 3 h, while the bacterial suspension was taken for evaluating the total and viable cell numbers present in the culture broth, the soaked film was carefully taken out of the suspension by the sterile forceps and immediately immersed into 2.5% glutaraldehyde solution (by weight). The bacteria adhered on the film surface were then fixed in the glutaraldehyde solution at 4°C for 24 h. After that, glutaraldehyde solution was removed and the film was rinsed by PBS followed by a stepwise dehydration with 30%, 50%, 70%, 90%, and 100% ethanol in water for 10 min interval. The film were then dried and sputter-coated with a thin film of gold before being characterized by scanning electron microscopy (SEM) (JEOL Model JSM-6400 electron microscope (Japan)).

3.3.4 Analytical methods

3.3.4.1 Proton nuclear magnetic resonance spectrometry (^{1}H NMR)

Proton (^{1}H) nuclear magnetic resonance analysis was used to characterize PLLA products. The sample was dissolved in chloroform-d ($CDCl_3$) and vortexed until clear solution was obtained. The NMR experiment was carried out by using Varian mercury-400 spectrometer ^{1}H NMR operating at 400 MHz. Chemical shifts (δ) were reported in parts per million (ppm) relative to the residual protonated solvent signal as a reference.

3.3.4.2 Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) was used to determined molecular weight of copolymer products ($\overline{M}_w$ and $\overline{M}_n$) and polydispersity index (PDI). The polymer sample (15 mg) was dissolved in tetrahydrofuran (THF) (3 mL) and filtered by syringe filter (diameter 13 mm, pore size 0.45 μm, nylon). The GPC chromatogram was obtained from Waters 150-CV chromatography equipped with PL-gel 10 μm mixed B 2 columns (MW resolving range = 500-10,000,000) at 35°C. THF was used as an eluant with the flow rate of 1.0 mL/min. Degassed THF mobile phase was passed through the column for 20 min before sample injection. 100 μL sample was injected and run for 40 min. Polystyrenes (MW = 5,460-1,290,000) were used as standards for calibration. The molecular weight was determined by a reflection index detector.

3.3.4.3 Differential scanning calorimetry (DSC)

Differential scanning calorimeter was determined by NETZSCH DSC204F1 Phoenix. This technique determines the physical property of semi-crystalline products. In this research, DSC technique was used to study glass transition temperature (T_g) of polymer products. Each sample was first heated from room temperature to 190°C and held at that temperature for 3 min. The sample was then

quenched to −30°C with liquid nitrogen and reheated at a rate of 10°C/min. The *Tg* values were taken as the midpoints of the specific heat jump.

3.3.4.4 Attenuated total reflection infrared (ATR-IR) spectroscopy

Attenuated total reflection infrared (ATR-IR) spectroscopy technique was used to analyze material surface. It was also suitable for characterization of materials which were either too thick or too strong absorbing to be analyzed by transmission spectroscopy. A single attenuated total reflection accessory with 45^{o}germanium (Ge) IRE (Spectra Tech, U.S.A.) and a variable angle reflection accessory (seagull^{TM}, Harrick Scientific, U.S.A.) with a hemispherical Ge IRE were employed for all ATR spectral acquisitions. All spectra were collected at a resolution of 4 cm^{-1} and 128 scans using a NICOLET 6700 FT-IR spectrometer with CONTINUμM IR Microscope equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride (MCT) detector.

3.3.4.5 Tensile property

The mechanical properties of films were studied. The tensile strength (TS), Young's modulus (Y) and percent elongation at break (%E) of the film samples were measured by Universal testing machine (UTM) (Figure 3.1). The film sample (0.5 × 4 in^2) was maintained in the desiccator prior to the test. The width and the thickness of the film were measured prior to testing. The test was carried out according to ASTM D 882 standard method, at 25^{o}C and 50% humidity, with crosshead speed of 12.5 mm/min, and load cell of 5.0 kN.

Figure 3.1 Universal testing machine (UTM)

3.3.4.6 In vitro tissue adhesion

The adhesive strength was tested by T-peel test. The adhesive strength test was performed *in vitro* using 30 µm thick polyamide (6/6 Nylon) films as a substrate. The film sample (2.5×5 cm^2) was adhered to substrate and cut into 2.5×10 cm^2. The tests (T-Peel Test ASTM D 1876) were performed using an Instron machine (model 5583) (Figure 3.2) and Adhesive Failure Strength (AFS) values were determined and reported in Newton per cm width units (N/cm width). The measurements were performed at a 30 mm/min crosshead speed at 37°C. Each test was repeated three times.

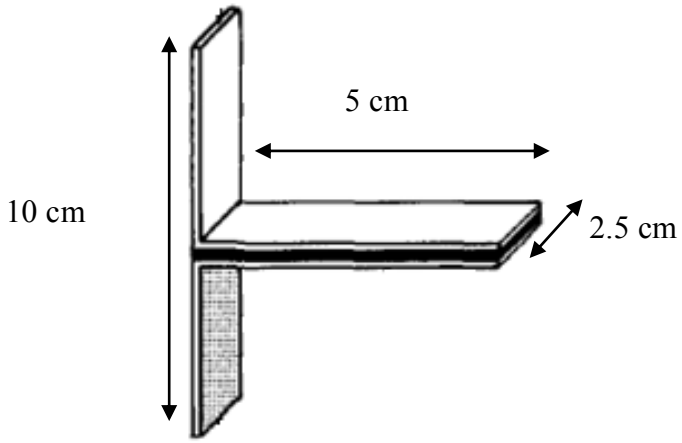

Figure 3.2 T-peel specimens.

3.3.4.7 Swelling property

Film swelling property was determined by measuring the water absorption content. The film was cut into 2×2 cm^2 and weighed (W_i) before immerging into 50 mL phosphate buffer saline (PBS) pH 7.4 at ambient temperature. After soaking, the film was removed and the free water on the film surface was carefully removed by drying between filter papers. The film was then weighed (W_o). The degree of swelling was calculated as follow:

$$\%swelling = \frac{W_i - W_o}{W_o} \times 100$$

3.3.4.8 In vitro doxycycline release study

The *in vitro* drug release of doxycycline was investigated. Drug loaded film square films (30 mm × 30 mm) were cut from dry films. Each film was immersed into the flask containing 50 mL phosphate buffer saline (PBS) (pH 7.4). The flask was incubated in the shaker at 37±1°C and 110 rpm. At the end of the predetermined time intervals, 3 mL sample was taken from the flask and the equal volume of fresh PBS was replaced into the flask in order to maintain a constant volume of medium during

the test. The solution sample was centrifuged and diluted to a suitable concentration if necessary. The UV absorption was measured by UV-VIS spectrophotometer at 274 nm. All experiments were performed in triplicate. The amount of doxycycline released was calculated by interpolation from a calibration curve containing various concentrations of doxycycline. A cumulative correction was made for the previously removed sample to determine the total amount of drug release.

The absorbance of standard solutions was determined by UV-VIS spectrophotometer at 274 nm. The PBS was used as a reference solution. Each concentration was determined in triplicates. The absorbance and calibration curve of TCH in PBS pH 7.4 are shown in Table C1 and Figure C1 (Appendix C), respectively.

3.3.4.9 In vitro MSG release study

In vitro release of MSG was investigated together with *in vitro* doxycycline release. HPLC was used to analyze the concentration of monosodium glutamate. The standard solution containing L-glutamic acid at various concentrations (0.25, 0.50, 1.00, 1.50, and 2.00 g/L) were injected as the references to determine the sample concentration. Samples were centrifuged, diluted, and filtered through cellulose acetate membrane. 15 µL diluted particles-free samples were injected into an analysis column (Sumi Chiral OA-5000; 150 mm x 4.6 mm) maintained at 40°C in a column oven (Shimadzu,CTO-10A) and 2mM $CuSO_4$ in H_2O/Methanol at the ratio of 95/5 was used as an eluant at 0.7 mL/min flow rate. An UV detector (Shimadzu, SPD-10A) was set to detect the L-glutamic acid at 254 nm.

3.3.4.10 The water vapor transmission rate (WVTR)

The borosilicate glass bottle was filled with anhydrous calcium chloride. The film was tied onto the mouth of the bottles and a container with film was weighte (w_0). The bottle was placed into a desiccators containing saturated sodium chloride solution (65% ± 5% relative humidity [RH]) and stored in an oven (maintained at

37ºC ± 1ºC. The average area available for vapor permeation was set at 8 cm^2 (A). After 24 h, the container was weighed (w_i) with a weighing balance 3 times. WVTR was calculated as follow:

$$WVTR = \frac{w_i - w_o}{A}$$

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preparation of CHI/PLA/PCL blended film

4.1.1 PLLA synthesis

PLLA synthesis by direct polycondensation was divided in two steps. The first step was to obtain a highly purified L-lactic acid. In the second step, PLLA was produced by direct polycondensation in diphenyl ether with a metal catalyst. L-lactic acid solution was heated at 140°C under N_2 atmosphere for 2 h. Water was removed from the reaction mixture by applying high vacuum pressure for 30 min. After that the reaction mixture was heated to 160°C until no condensate was present. The product in the first step was a low molecular weight poly (L-lactic acid), so called LPLA. The Experimental set up for direct polycondensation PLLA synthesis is shown in Figure 4.1(a).

LPLA was heated to 140°C with catalyst under vacuum pressure. PLA crystal was collected by dissolving in $CHCl_3$, and precipitated with methanol. After that it was dried in vacuum at 50°C for 24 h. The resulting PLLA powder is shown in Figure 4.1(b).

(a) **(b)**

Figure 4.1 (a) Experimental set up for direct polycondensation PLLA synthesis
(b) PLLA by direct poly condensation.

Metal catalysts such as $Sb(OAc)_2$ and $Co(OAc)_2$ are commonly used in the polymerization of polyethyleneterephthalate (PET). Polycondensation of PLLA requires the similar mechanism and catalysts to PET synthesis which results in water as a major product from the combination of 2 carboxylic groups. In addition, $Sn(Oct)_2$, a commercial catalyst for ring opening polymerization was chosen to compare with the above 2 mentioned catalysts.

PLLA synthesis was carried out by direct polycondensation in diphenyl ether solution at various temperatures and metal catalyst. The M_n and Mw of synthesized PLLA are shown in Table 4.1. From the results of M_n and Mw, it was found that among 3 catalysts studied, $Sb(OAc)_2$ gave a highest molecular weight of 29,807 at 72 h. Therefore, $Sb(OAc)_2$ is the appropriate catalyst for direct polycondensation of PLLA synthesis in this work.

Table 4.1 Molecular weights and polydispersity index of PLLA obtained by direct polycondensation at 140°C with $Sb(OAc)_2$, $Co(OAc)_2$ and $Sn(Oct)_2$ as a catalyst.

Time	**$Sb(OAc)_2$**			**$Co(OAc)_2$**			**$Sn(Oct)_2$**		
(h)	M_n	M_w	*PDI*	M_n	M_w	*PDI*	M_n	M_w	*PDI*
24	5,452	6,690	1.22	2,317	2,563	1.10	3,983	5,042	1.27
48	5,828	11,546	1.98	2,320	2,630	1.13	9,637	13,367	1.39
72	23,706	29,807	1.25	2,741	3,272	1.19	9,732	14,544	1.49
96	13,570	28,437	2.10	3,252	3,854	1.19	15,531	20,664	1.33

Direct polycondensation of PLLA requires high reaction temperature because of the high melting point of diphenyl ether (121°C at 10 mmHg). The molecular weight of the resultant PLLA product was in between 9,500 and 14,000 at the reaction temperatures of 160°C and 180°C. The reaction temperatures were used in this work were higher than the melting point of PLLA (T_m = 146.4°C). One should be taken into account that high temperature presumably leads to faster reaction and somewhat more prevalence to thermal degradation which results in the low molecular weight PLLA product. In this work, the highest MW of 30,000 PLLA was achieved when the reaction was carried out at 140°C for 72 h. The M_n and M_w of synthesized PLLA are shown in Table 4.2.

Table 4.2 Molecular weights and polydispersity index of PLLA obtained by direct polycondensation at 140-200°C with $Sb(OAc)_2$ as a catalyst.

Time	**140°C**			**160°C**			**200°C**		
(h)	M_n	M_w	*PDI*	M_n	M_w	*PDI*	M_n	M_w	*PDI*
24	5,452	6,690	1.22	6,142	8,331	1.36	2,479	2,835	1.14
48	5,828	11,546	1.98	6,006	9,269	1.54	3,838	4,395	1.32
72	23,706	29,807	1.25	8,864	12,082	1.36	6,058	7,346	1.22
96	13,570	28,437	2.10	11,067	14,408	1.31	8,017	9,506	1.43

^{1}H-NMR spectra of synthesized PLLA are shown in Figure 4.2. In the spectra, the peaks appearing at 5.1 – 5.2 ppm are methine protons (-$\underline{CH}CH_3$) in PLLA repeating unit while the peaks at 1.5 ppm are methyl protons (-$CH\underline{CH_3}$) in the repeating unit of PLLA. A little interference peak of the residual lactic acid appear at 1.0 – 1.3 ppm.

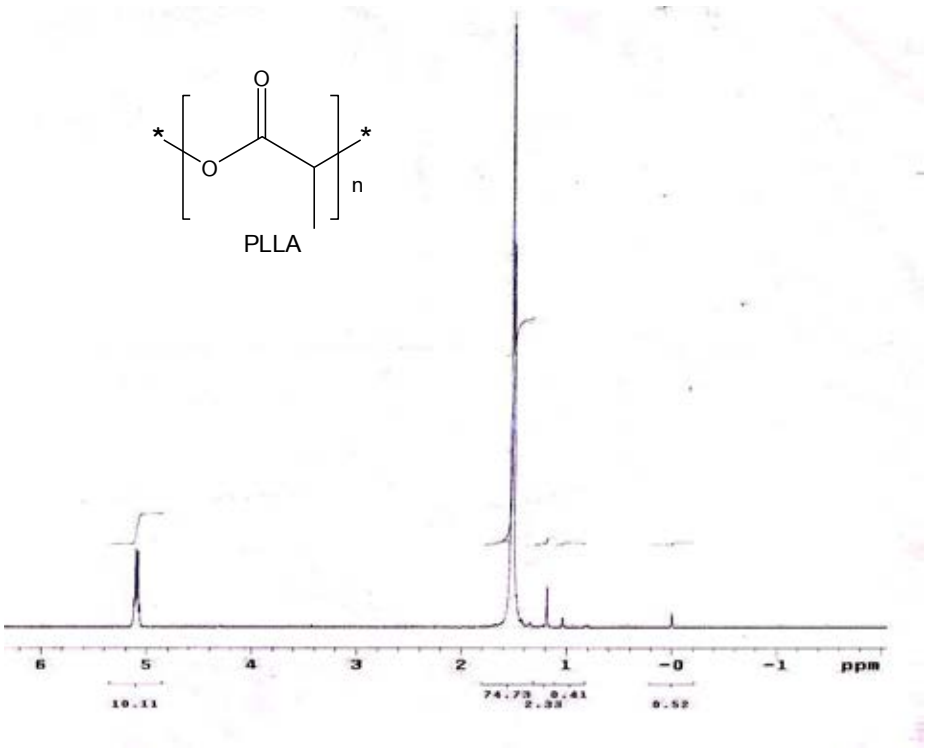

Figure 4.2 ^{1}H NMR of PLLA prepared by direct polycondensation at 140^{o}C using $Sb(OAc)_2$ as a catalyst.

The glass transition temperature (T_g) of highest molecular weight of synthesized PLLA (29,807) was measured by DSC. The typical PLLA glass transition temperature (T_g) ranges from 50°C to 80°C, whereas its melting temperature ranges from 130°C to 180°C (Avérous .,2008) The DSC chromatogram of obtained PLLA is shown in Figure 4.3. In this chromatogram, the T_g of the sample is at 55.6°C whereas the melting temperature is at 146.4 °C.

Low molecular weight of synthesized PLLA is appropriate to film formation and suitable candidates for utilizing as tissue adhesives. This is because of low molecular weight polymers have much lower viscosities and display a sharp decrease in viscosity at a temperature slightly above their typically low glass transition temperatures and also enhanced conformability and better attachment to the tissues. Due to this behavior, low molecular weight polymers were selected.

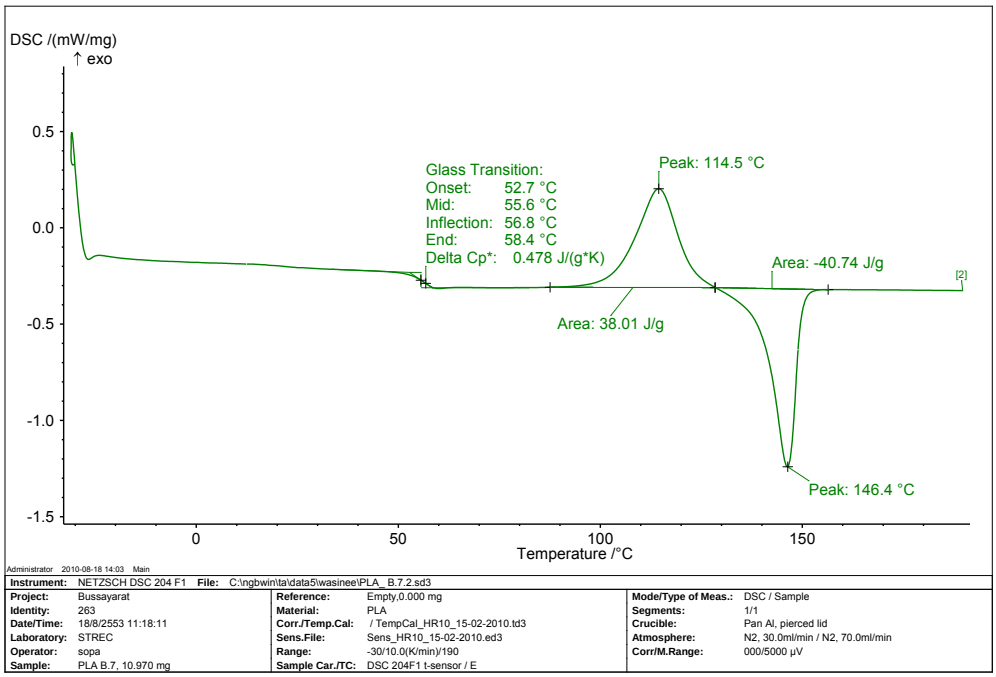

Figure 4.3 DSC chromatogram of PLLA prepared by direct polycondensation at 140^{o}C using $Sb(OAc)_2$ as a catalyst.

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For the T_g of chitosan, the determined baseline step is small due to the molecular structure of chitosan consisting of rigid β-1,4-linked D-glucosamine units. As a result, the change in heat capacity corresponding to the change in specific volume (or molecular mobility) at T_g is equally small (Suyatma et al., 2004)

PCL is a semicrystalline polymer with a melting point of 61°C and a glass transition temperature of -62.5°C (Table 4.3). It was expected that T_g and T_m could be shown in DSC chromatogram of the blended CHI/PLA/PCL film. However, only Tm of PLA and PCL were detected while T_g of the blended film did not appear on the chromatogram. This is because the T_g of PLA (55.6°C) was overlapped with t_m of PCL (61°C). DSC chromatograms of CHI/PLA/PCL blend films are shown in Table 4.3 and Figure A23-A27 (Appendix A).

Table 4.3 Thermal properties of CHI film, CHI/PLA/PCL blend films with thedifferent PLA and PCL ratios, PCL powder and PLLA prepared bydirect polycondensation at 140^{o}C using $Sb(OAc)_2$ as a catalyst.

Film (CHI:PLA:PCL ratio)	**T_g (°C)**	**T_m (°C)**
Chiosan	N.D.	N.D.
PCL (powder)	-62.5	61
CHI/PLA/PCL (60:4:36)	N.D.	59.6 (PCL) 130.1 (PLA)
CHI/PLA/PCL (60:12:28)	N.D.	58.0 (PCL) 130.8 (PLA)
CHI/PLA/PCL (60:20:20)	N.D.	56.8 (PCL) 132.3 (PLA)
CHI/PLA/PCL (60:28:12)	N.D.	56.7 (PCL) 129.6 (PLA)
CHI/PLA/PCL (60:36:4)	N.D.	55.4 (PCL) 129.7 (PLA)
PLA (powder)	55.6	146.4

4.1.2 Film characteristics

In order to attain all required properties of wound dressing device, polymer blend is attractive since blending process can be easily done while the blend polymer acquires all desired properties (Sahoo et al., 2009). In this work, CHI, PLA, and PCL were selected for blending. CHI is an important polymer for wound dressing film as reported in many previous studies due to the good swelling degree and antimicrobial activity. PLA and PCL provide the superior physical properties to the film. PLA serves on the film strength while PCL helps in term of flexibility. However, blending CHI/PLA/PLA suffered from the immiscibility of the hydrophobic PLA/PCL organic phase into the hydrophilic CHI solution. To eliminate the problem, Gly or PEG were used as the surfactant in order to reduce the surface tension between organic and aqueous phases and to increase their miscibility (Chen *et al.*,2003).

Firstly, PLA/PCL blend was prepared by dissolving PLA and PCL in dichloromethane at the fixed weight ratio of 1:1 (50%:50%). CHI/PLA/PCL blend at 3 different weight ratios of CHI to PLA/PCL blend (30:70, 40:60, and 60:40) was prepared. PLA/PCL blend was gently dropped into 2% chitosan solution with the presence of glycerine or polyethylene glycol (PEG) at 0.5% wt as an emulsifier. It was found that CHI/PLA/PCL blend was more miscible with the presence of surfactant.

The ATR-IR spectra show the actual characteristics of CHI film, PLA/PCL blend film, and CHI/PLA/PCL blend film (Figure 4.4). It was found that the CHI/PLA/PCL blend film acquired all characteristics of the 3 homopolymers used (Figure 4.4 (c)). In the spectrum of PLA/PCL blend film, the C=O, C–O–C, and C–C peaks were clearly observed at 1750, 1175 and 1200 cm^{-1}, respectively (Figure 4.4 (b)). From the spectrum of CHI film, an amino group in CHI appeared at 1560 cm^{-1} and hydrogen bonded O–H stretch vibrational at 3400 cm^{-1}. In the C–H stretch region of spectrum, the higher intensity peak at 2923 cm^{-1} was assigned to an asymmetric CH_2 of CHI (Figure 4.4 (a)). It is clear that from the ATR-IR spectra of CHI/PLA/PCL blend film, all characteristic peaks of CHI and PLA/PCL appeared at 3400, 2900, 1750, and 1560 cm^{-1} indicating that the modified spontaneous emulsification-solvent diffusion technique was successfully used in preparing the CHI/PLA/PCL blend film that represents all major characteristics of the 3 polymers.

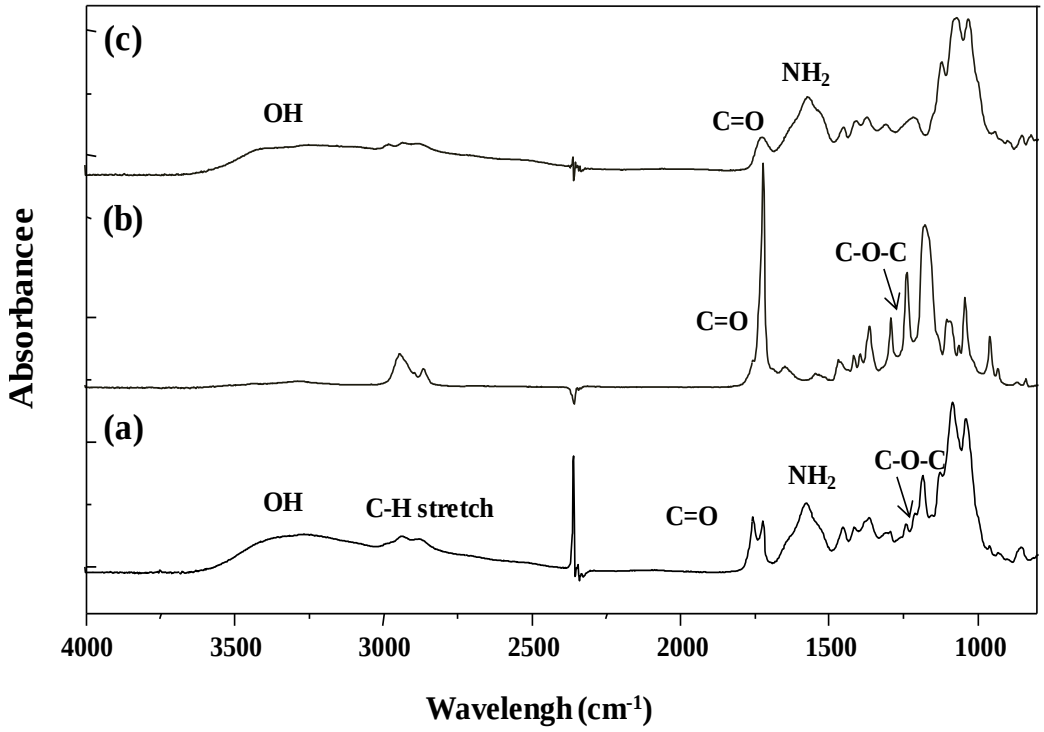


Figure 4.4 ATR-IR spectra of (a) CHI film, (b) PLA/PCL blend film, and (c) CHI/PLA/PCL blend film.

4.1.3 Film formation and swelling behavior of CHI/PLA/PCL blend film

It was found that when using Gly as the emulsifier, the obtained film was more transparent and easily peeled off from the petridish surface compared with the film prepared by using PEG as the emulsifier (data not shown). Figure 4.5 shows the swelling behavior of CHI/PLA/PCL blend films. It was observed that the blend films using PEG as the emulsifier was highly swollen and lost the stability after soaking in PBS buffer within the short period of time (less than 10 min). The swelling behavior of any polymer network depends on the nature of the polymer, polymer-solvent compatibility, and degree of cross linking. Besides, in the case of the ionic networks, mass transfer limitation and ionic interaction affect the swelling behavior (Korsmeyer et al., 1981). In this work, PEG or Gly were selected for cross linking CHI together with PLC/PCL blend. The degree of cross linking of the polymer blend eventually affected the swelling degree and film stability as observed from this study. Not only

the high swelling degree but the stability also play important role in making the wound dressing device. Due to Gly could provide the superior film stability compared with PEG; therefore, it was selected as the emulsifier throughout this study.

It was also found that increasing the CHI ratio in the CHI/PLA/PCL blend film while the ratios of PLA and PCL were kept constant enhanced the film swelling degree and improved the film stability (Figure 4.5). This was because of the better swelling property of CHI but the fragility of PLA and PCL. Total film weight also played role in the swelling property of CHI/PLA/PCL film. Increasing the film weight from 0.5 g to 1.0 g yielded more than twice increasing the swelling degree of the CHI/PLA/PCL (60:20:20) blend film with the film thickness of 0.15 – 0.20 mm. Therefore, the total weight of 1.0 g was selected for film preparation in further studies in this work.

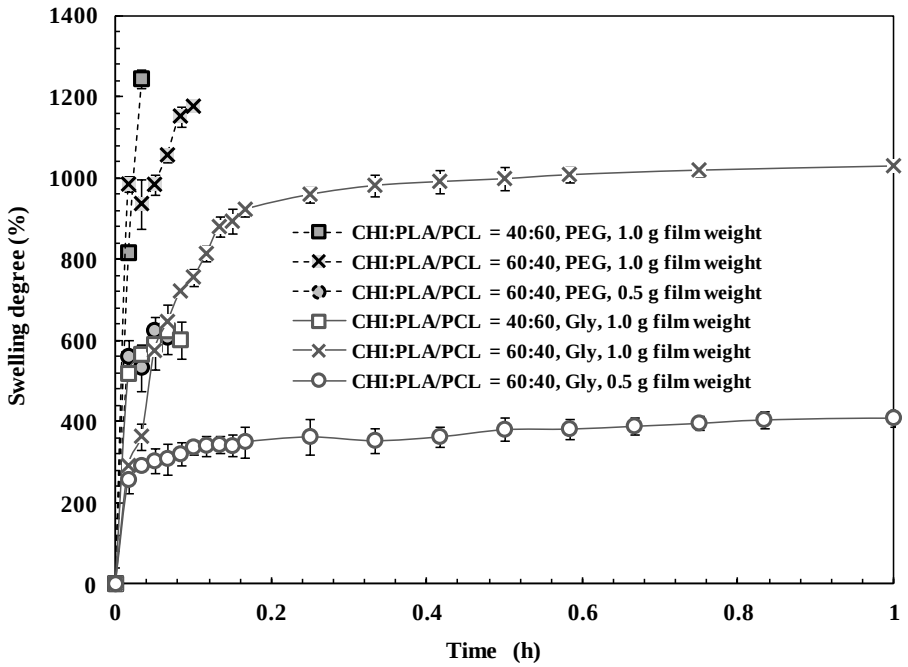


Figure 4.5 Swelling behavior of CHI/PLA/PCL blend films in PBS buffer (pH 7.4). PLA and PCL were initially blended at the ratio of 50:50 before they were mixed with CHI solution at the different ratios with the presence of Gly or PEG at 0.5 wt%. The polymer blend solution was then casted by the modified spontaneous emulsification-solvent diffusion method at 2 different weights of solution.

The appearance of the blend film with glycerine and PEG400 as the surfactant was shown in Figure 4.6. It was found that the CHI/PLA/PCL blend film with the presence of glycerine was more transparent, easily peeled out from petri dish. In addition, high swelling degree was observed in this film compared with the one using PEG400 as the surfactant. The total weight of CHI/PLA/PCL blend film of 1.0 g with the thickness of 0.15 – 0.20 mm (CHI/PLA/PCL of 0.6 g: 0.2 g: 0.2 g) gave the most suitable film properties.

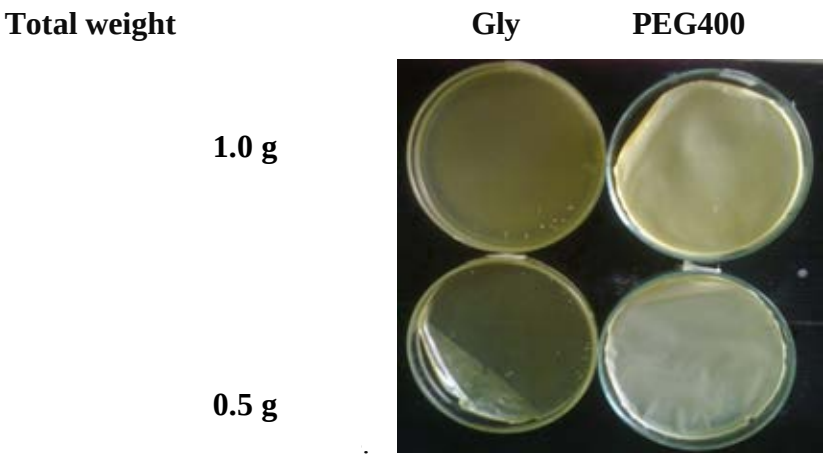


Figure 4.6 CHI/PLA/PCL film with the present of glycerine and PEG400.

4.1.4 Improved film mechanical properties

As mentioned earlier that CHI provides the good swelling property while PLA and PCL give the superior mechanical properties to the film. The good swelling property is required in wound dressing device since the film with the high swelling degree serves on the high release rate of the active compound of interest to the injury site; thus, facilitating the clotting process. Film stiffness but flexibility is another property required in preparing wound dressing device so that the film can serve as the support that can withstand the blood pressure from bleeding at the opened wound. Improved film stiffness while maintaining the flexibility could be obtained by varying the ratio of PLA and PCL with the fixed CHI ratio. When varying the ratios of PLA and PCL while keeping CHI ratio constant, the swelling degrees of those prepared

films were similar (Table 4.4). However, increasing the PLA content with the reduction in PCL content led to the larger tensile values but the lower elongation percentages (Table 4.4). This can be explained by the natural characteristic of pure PLA is hard and brittle while PCL could provide the elasticity property to the film (Chen et al.,2003). Compared with the blend films at all ratios studied, virtue CHI film gave the highest swelling degree and tensile values; nevertheless, the elongation property was the poorest.

The results of *in vitro* tissue adhesion observed from T-peel test on all prepared films imply that PCL promoted the elasticity of the blend film as increasing the PCL content resulted in the larger T-peel stress (Table 4.4). Tanveer et al (2000) reported that increasing the flexibility of the blend film helps improve the surface interaction between the film and the tissue. Therefore, more flexible film obtained in the blend film at the higher PCL content could yield the better surface adhesion.

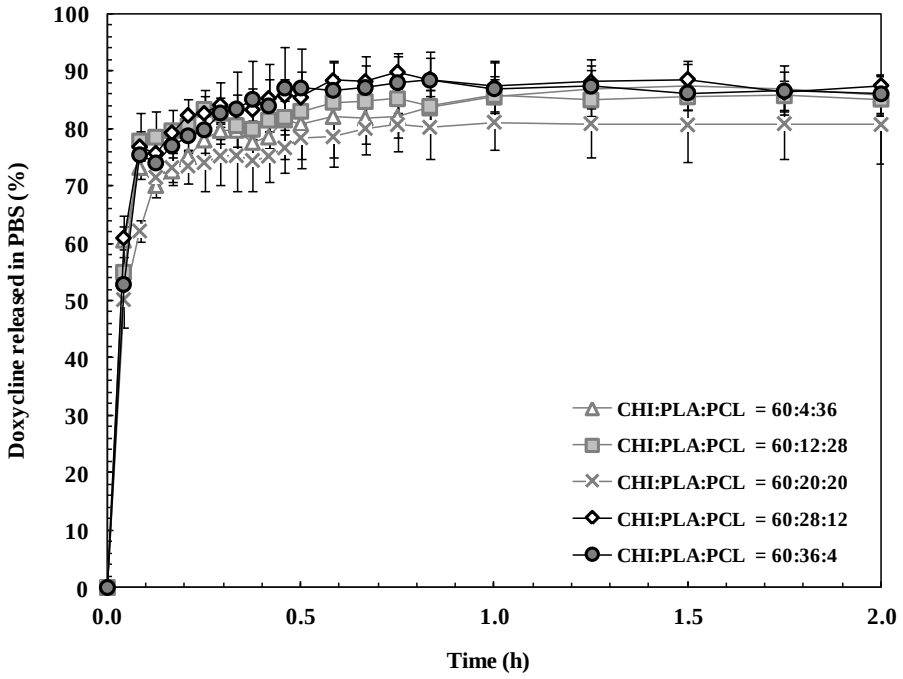


Figure 4.7 Swelling behavior of CHI/PLA/PCL blend films with the presence of Gly at 0.5 wt% with the different PLA and PCL ratios in PBS buffer (pH 7.4). The total film weight was set at 1.0 g.

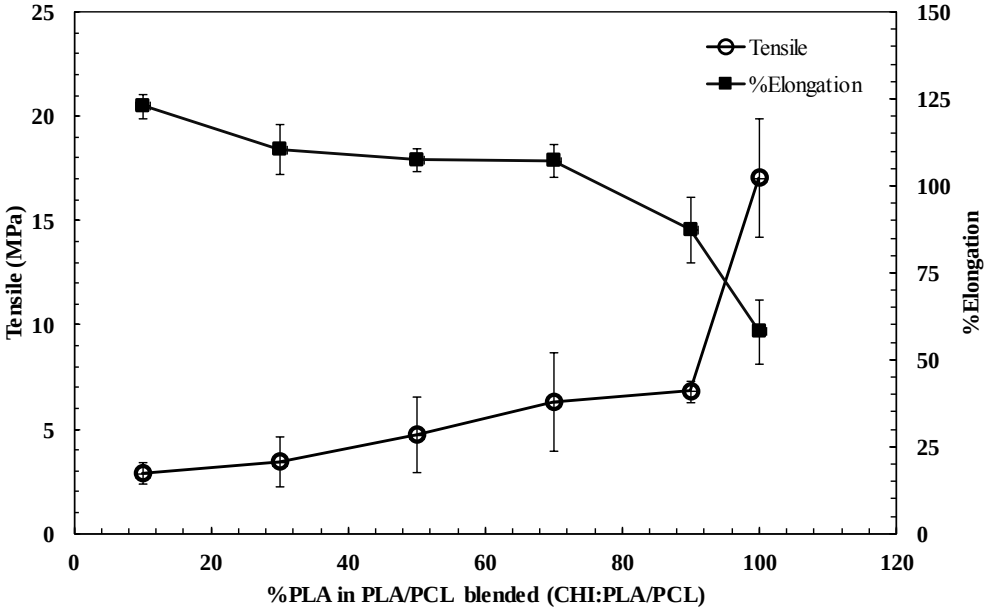


Figure 4.8 Tensile strength and % elongation of CHI/PLA/PCL film with a different weight ratio of PLA/PCL.

Table 4.4 The properties of CHI/PLA/PCL blend films with the different PLA and PCL ratios. The total film weight was set at 1.0 g.

Film (CHI:PLA:PCL ratio)	Film Thickness (mm)	Swelling degree (%)	Water-vapor transmission rate (g/m^2/day)	Tensile (MPa)	Elongation (%)	T-peel (N/cm)
CHI	0.270	1,545.03±34.01	6,050±15.03	17.069±2.819	58.223±9.167	0.0428±0.0003
CHI/PLA/PCL (60:4:36)	0.180	991.15±52.83	3,750±17.01	2.898±0.512	122.99±3.605	0.0418±0.0006
CHI/PLA/PCL (60:12:28)	0.172	1,007.12±1.84	3,562±12.05	4.127±1.177	105.221±7.168	0.0355±0.0007
CHI/PLA/PCL (60:20:20)	0.170	1,029.44±15.64	3,325±5.08	4.749±1.807	107.445±3.339	0.0336±0.0008
CHI/PLA/PCL (60:28:12)	0.160	1,004.65±22.51	2,875±23.04	6.313±2.367	107.332±4.583	0.0331±0.0005
CHI/PLA/PCL (60:36:4)	0.158	1,036.96±13.28	2,862±19.09	6.821±0.506	87.83±9.457	0.0256±0.0023

4.1.5 Water – vapor transmission rate (WVTR) and controlled release of CHI/PLA/PCL blend film

WVTR measures a capacity of the water vapor passing through a substance of interest. The ability to transport water vapor is an important function of the wound dressing device. The excessive WVTR may lead to wound dehydration and strong adhesion of the dressing onto the wound surface leading to the second injury when removing the device from the wound. On the other hand, the low WVTR causes maceration of healthy surrounding tissue and builds up a back pressure and pain to the patient. It also leads to the leakage and breakage at the wound area nearby the dressing edge which eventually results in dehydration and bacterial infection (Queen et al., 1987). It has been claimed that the ideal value of WVTR of the wound dressing device falls approximately within the range of 2,000 – 2,500 $g/m^2 \cdot day$ (Boateng et al., 2008). In this study, the WVTR value of the CHI/PLA/PCL blend film was examined. From Table 1, it was found that the WVTR value of the virgin CHI film was the highest (6,050 $g/m^2 \cdot day$). Increasing PLA content yielded the lower WVTR value of the blend film. The blend films containing CHI:PLA:PCL at the ratios of 60:28:12 and 60:36:4 gave the WVTR values approaching the desirable WVTR value of the wound dressing device; thus, these 2 compositions were preferred in device fabrication.

Doxycycline controlled release from the CHI/PLA/PCL blend film in the PBS buffer (pH 7.4) was investigated (Figure 4.9). The similar doxycycline release profiles observed from all blend films appeared into 2 stages. The initial stage was a rapid release that occurred within the first 30 minutes. Later it was slowly released from the blend film until approaching the equilibrium. In addition, it was observed that all blend films prepared in this study gave the high doxycycline release rate (>80%).

According to the results shown in Table 4.4, the CHI/PLA/PCL blend film containing CHI of 60%, PLA of 28%, and PCL of 12% at the total film weight of 1.0 g gave the superior tensile and adhesive strengths, the good swelling and WVTR degrees, and the high doxycycline release rate. Therefore, the blend film at this composition was prepared for the study on in vitro blood clotting and in vitro antimicrobial activity.

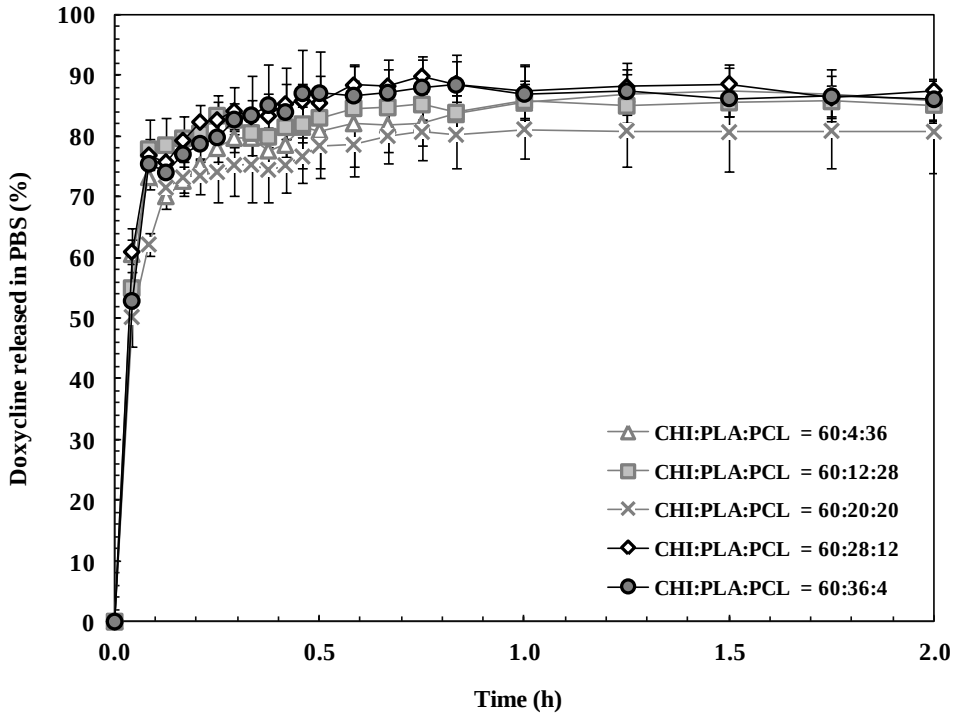


Figure 4.9 Doxycycline dissolution from the CHI/PLA/PCL blend films with different PLA and PCL contents in PBS buffer (pH 7.4) at 37°C.

4.1.6 Effect of monosodium glutamate (MSG) and oleanolic acid on human blood clotting

Human blood plasma was prepared from the whole human blood supplied by the Thai Red Cross Society and the composition of human blood is shown in Figure 4.10. Human blood plasma was obtained by centrifugation of citrated blood for 15 min at 1,500g.

Monosodium glutamate (MSG) and oleanolic acid were studied for their application as the active ingredients to accelerate blood clotting process. Model MSG and oleanolic acid solution were tested at various concentrations with human blood plasma to investigate their concentration effect on the Prothrombin time (PT) and the activated partial thromboplastin time (aPTT).

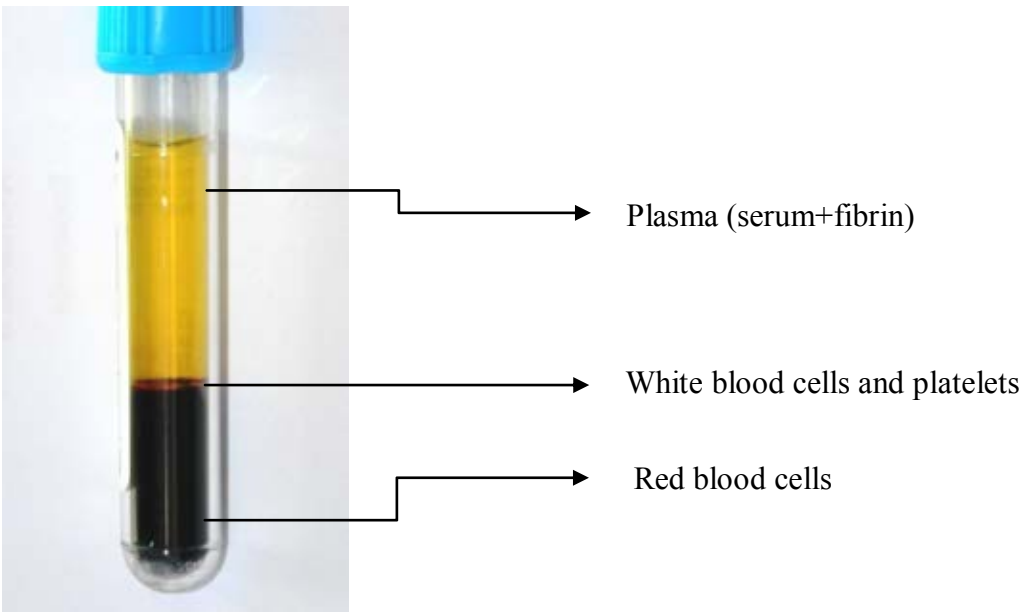


Figure 4.10 Composition of human whole blood with sodium citrate as anticoagulant.

Oleanolic acid not soluble in water, it can be solute in organic solvent such as buthanol and methanol, and in this work it was prepared in methanol. The solution of oleanolic acid was tested with PT and aPTT reagents and it was found that the testing reagents were precipitated with oleanolic acid solution. According to *in vitro* blood clotting test of oleanolic acid, it is indicated that oleanolic acid is not suitable to be used as the active ingredient in the wound dressing.

In vitro human blood clotting was studied. The prothrombin time (PT), the activated partial thromboplastin time (aPTT) as well as fibrin formation was observed when MSG solution at various concentrations (from free MSG plasma to plasma containing saturated MSG) was added into the plasma (Figure 4.12). It was found that PT and aPTT times could not be detected as the result of fibrin formation at the MSG concentration of 200 g/L or higher (until reaching MSG saturation in plasma). At the low MSG concentrations (0 – 35 g/L) tested, increasing the MSG concentration lowered the PT and aPTT times compared with the control. At the MSG concentration higher than 35 g/L, the PT and aPTT times increased with the increasing MSG concentration. When increasing the tested MSG concentration approaching 200 g/L or

higher, fibrin formation was observed as can be seen from the scanning electron micrograph (Figure 4.13). The evidence of the decreasing PTT and aPTT times at MSG concentration ≤ 35 g/L, the increasing PTT and aPTT times at MSG concentration ≥ 50 g/L, and the fibrin formation at MSG concentration ≥ 200 g/L implied that MSG could be used at the low concentration to promote the clotting process. While the concentration higher than 35 g/L induced rapid fibrin formation which eventually led to strong adhesion and the second injury when the wound dressing device was taken off for further medical care after first aid.

4.1.7 Blood clotting test on Doxy/MSG loaded CHI/PLA/PCL blend film

Controlled release of doxycycline and MSG from the Doxycycline/MSG loaded CHI/PLA/PCL blend film was observed. The CHI/PLA/PCL blend film containing 60% CHI, 28% PLA, and 12% PCL at the total weight of 1.0 g was loaded with doxycycline (0.03 g) and MSG (0.2g). The dissolution profiles were shown in Figure 4.11. Similar dissolution profiles were observed in the case of doxycycline and MSG, i.e., during the initial phase both were rapidly released within 30 min. Later, they were slowly released until reaching equilibrium. Both doxycycline and MSG loaded onto the blend film were almost completely released; thus, confirming that this prepared doxycycline/MSG loaded CHI/PLA/PCL blend film could be used for enhancing blood clotting process.

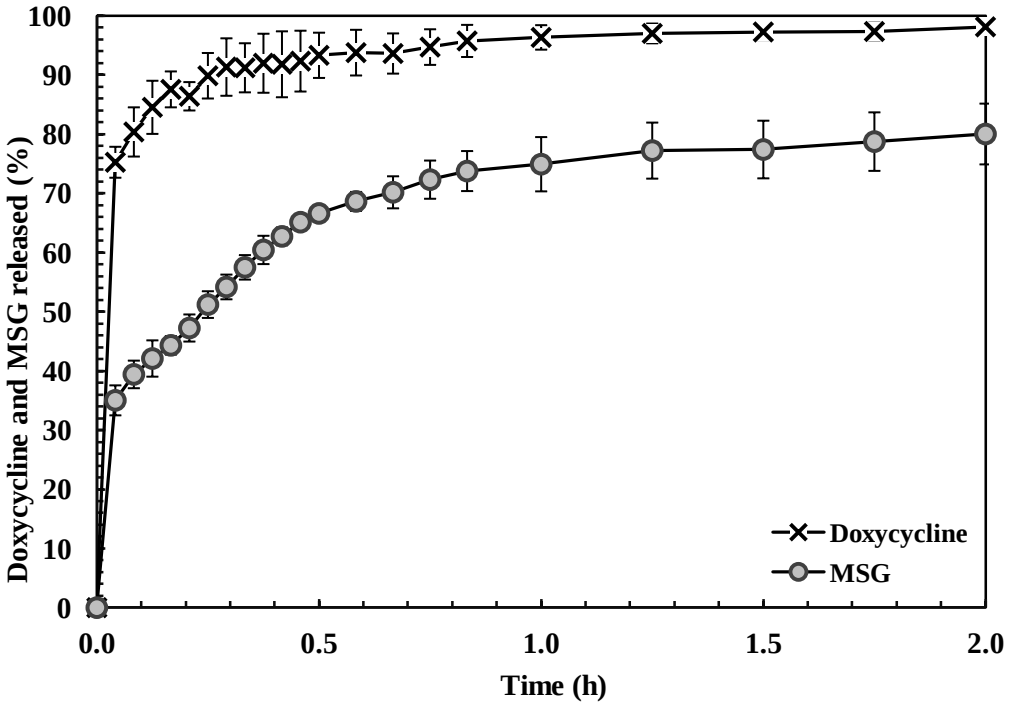


Figure4.11 Doxycycline and MSG dissolution from the CHI/PLA/PCL blend film at the ratio of CHI:PLA:PCL of 60:28:12 in PBS buffer (pH 7.4) at 37°C.

Figure 4.14 shows the scanning electron micrographs representing red blood cell and platelet adhesion onto various film surfaces. It was found that the virgin CHI film, CHI/PLA/PCL blend film, Doxycycline/MSG loaded CHI/PLA/PCL blend film, and the commercial dressing device exhibited a strong hemostatic activity as observed from a large amount of red blood cells aggregated onto these film surfaces. Whereas the virgin PLA film possessed the weak hemostatic activity (Figure 4.14(b)). The presence of such individual red blood cells (or erythrocytes) was perhaps due to the mechanical retention of the cells into the highly porous structure of PLA film. In contrast, the films containing CHI acquired the NH_2 functional group; thus initiated the strong electrostatic interaction with the negatively charged red blood cells (Wang Q.Z. et al., 2008, Edwards et al., 2009). This resulted in more red blood cells aggregated onto these films (Figure 4.14(a), (c), and (d)). Although red blood cells were strongly aggregated onto the surface of all the tested films containing CHI, the evidence of fibrin formation was observed only on the surface of the Doxycycline/MSG loaded CHI/PLA/PCL blend film. This result confirmed that MSG

induced fibrin formation in blood coagulation process. In the case of platelets adhesion onto the film surface, it was found that a large amount of platelets were adhered onto the surfaces of the films containing CHI and the commercial dressing device while only little platelets were observed on the PLA film surface.

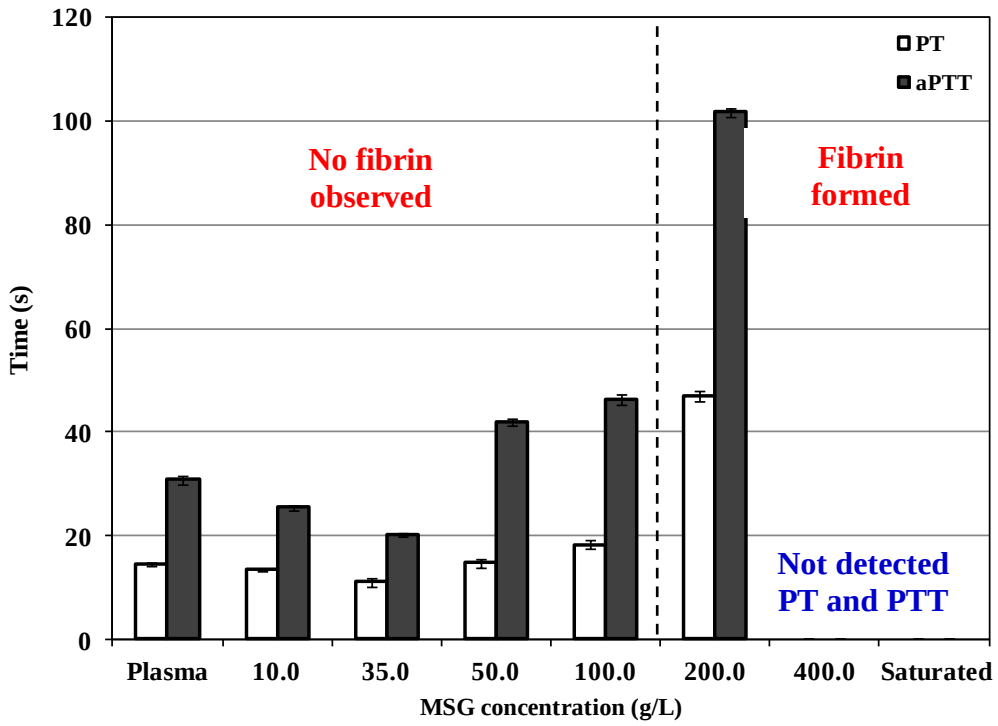
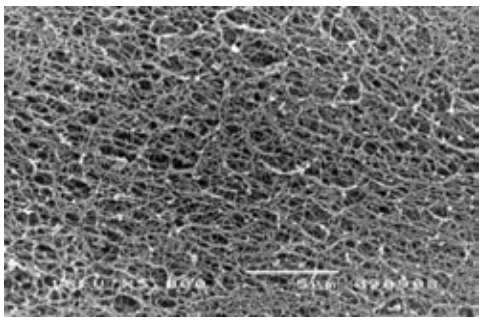


Figure 4.12 Concentration effect of monosodium glutamate (MSG) on in vitro human blood clotting process.

(a)



(b)

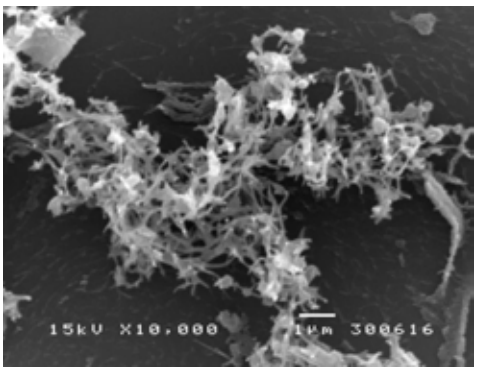


Figure 4.13 Scanning electron micrograph indicating fibrin formation appeared after an addition of monosodium glutamate (MSG) at the concentration approaching 200 g/L or higher into the human blood plasma at (a) the magnification of 5,000 and (b) the magnification of 10,000.

Blood clotting is a complex process, occupying many factors present in the plasma and tissue. MSG promotes blood clotting process in 2 ways. Sodium (Na) ion in MSG activates protein clotting factor (Factor IXa) by working with Factor VIIIa to stimulate the blood clotting mechanism. While glutamate group in MSG accelerates vitamin K by reducing it into an active oxidizing form which is readily to stimulate Factor II (Prothrombin), Factor VII, Factor IX, and Factor X required in blood clotting mechanism (Bowen., 1999; Higdon., 2004; Tollefsen., 2006). From the blood clotting results, it could be concluded that MSG accelerated blood coagulation and induced fibrin formation while a cationic nature of CHI in the CHI/PLA/PCL blend film interacted with the negatively charged biological molecules on the red blood cells and platelets surfaces. Therefore, the prepared doxycycline/MSG loaded CHI/PLA/PCL blend film is proper for making wound dressing device.

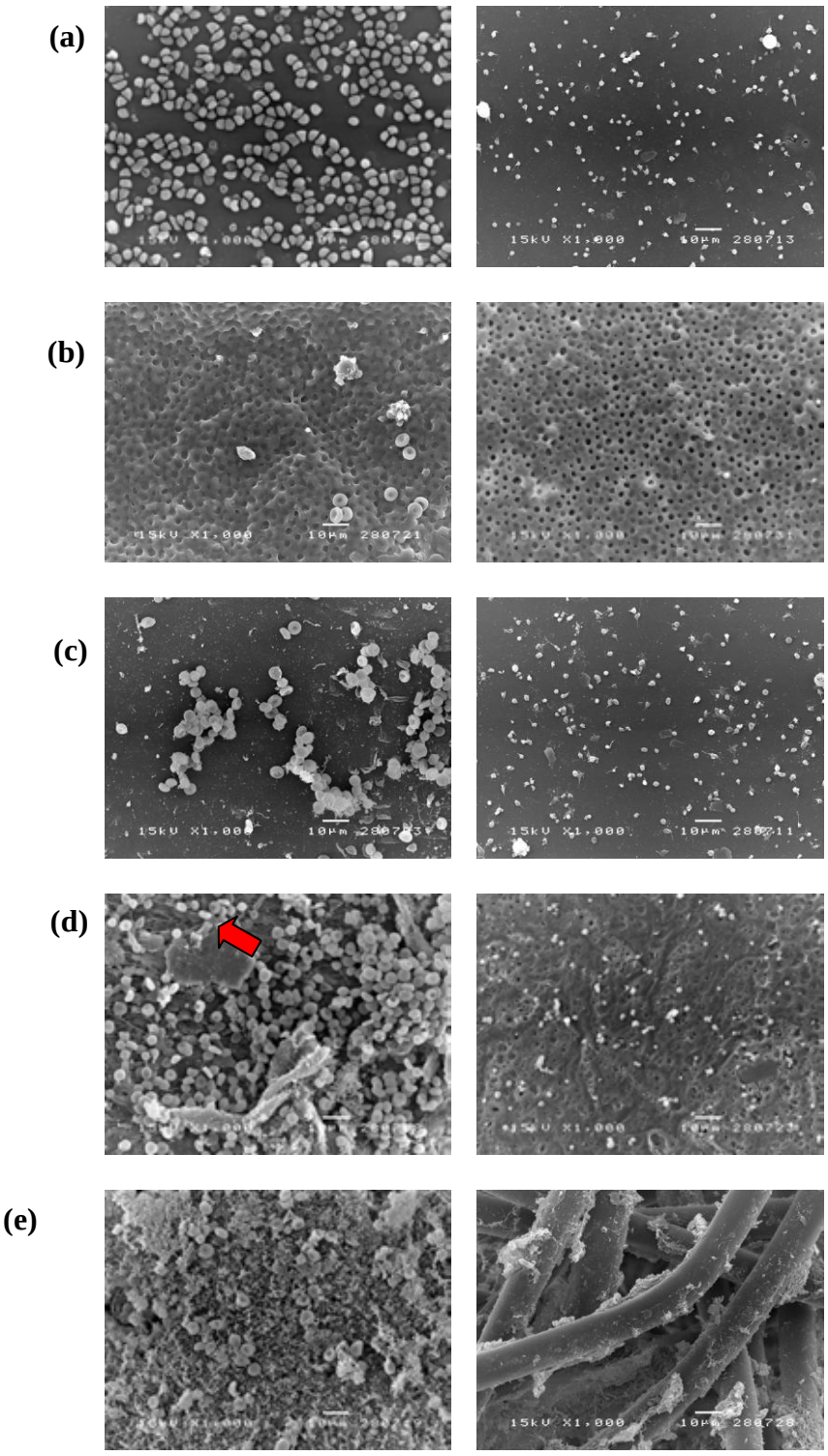


Figure 4.14 Scanning electron micrographs of red blood cell (on the left) and platelet (on the right) adhesion onto (a) CHI film; (b) PLA film; (c) CHI/PLA/PCL (60/28/12) blend film; (d) Doxycycline/MSG (0.03 g/1.00 g) loaded CHI/PLA/PCL (60/28/12) blend film; and (e) the commercial Boots stop bleeding fast dressing.

4.1.8 Antibacterial activity of the doxycycline/MSG loaded CHI/PLA/PCL blend film

Bacterial contamination to the wound is one of the serious problems that threaten patient's life. In this study, different films prepared were tested against the selected bacteria for their antibacterial activity. Figure 4.15 shows the growth kinetics patterns of *S. aureus* and *E. coli* grown inn TSB media with the presence of different films prepared. Similar growth pattern to the control bacterial culture was observed in the cultures with the presence of commercial film, PLA film, CHI/PLA/PCL film, and neutralized CHI film for both *S. aureus* and *E. coli*. Delayed growth was observed in the culture with the non-neutralized CHI film while doxycycline/MSG loaded CHI/PLA/PCL film shows a significant antibacterial activity.

Bacterial adhesion on the film surface can be plausibly explained by these 2 phenomena, i.e., the physical attachment due to the roughness of the film and the ionic (charge-charge) interaction. Due to the strong hydrophobicity of the commercial dressing device made from micro-dispersed oxidized cellulose, the PLA film, and the CHI/PLA/PCL film, this limited the bacterial surface adhesion onto the film surface. As a result, these 3 films did not show any effect on bacterial cell growth as observed from the similar growth pattern as observed in the typical bacterial culture in the control condition.

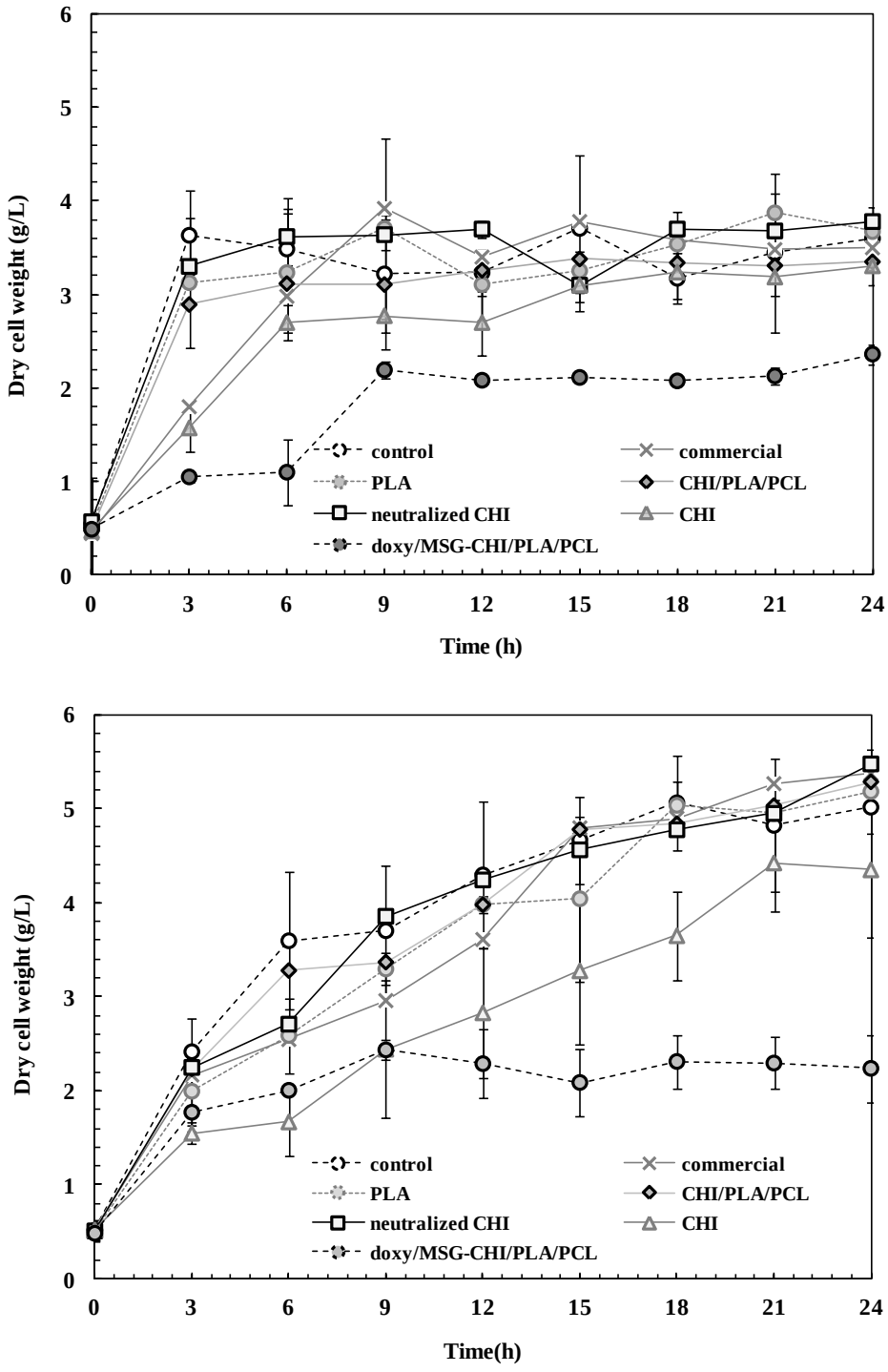


Figure 4.15 Growth kinetics of (a) *S. aureus* and (b) *E. coli* when different polymeric films were immersed into the bacterial culture broth compared with the control bacterial culture where no film was present.

Although it was claimed that CHI possessed the antibacterial activity; however, the mechanism has not yet to be confirmed. In this study, it was found that when neutralized CHI film was present in the bacterial culture, this film did not show any antibacterial activity as observed in the cultures with the presence of the commercial, PLA, and CHI/PLA/PCL films. Due to the working pH of the bacterial culture was at 7.0, the amino groups in CHI were not readily in the protonated form (NH_3^+). Therefore, there was no affinity for bacterial binding onto the neutralized CHI surface. On the other hand, when immersing the CHI film without neutralization into the bacterial culture, the nature of polycationic ion (NH_3^+) of the amino group that initiated the binding with the cell membrane by means of electrostatic attraction with the microbial cell membrane that possessed some negative charges. The surface interaction might cause the change in transporting the necessary nutrients from the broth into the cells or secreting the wastes inside the cells into the broth. The change in transport of nutrients and wastes throughout the cell membrane eventually led to the delayed growth as observed in Figure 8. The evidences of the delayed growth and the change in cell proliferation pattern were more profound as observed from the scanning electron micrographs of the CHI film that was exposed into the *E. coli* culture (Figure 4.16). Instead of normal cell division and proliferation, with the presence of CHI film in the culture, after cultivation for 3 h *E. coli* entered the filamentation stage which limited cell reproduction during this stage. The filamentation process is the anomalous growth of certain bacteria such as *E. coli* in which cells continue to elongate but do not divide (no septa formation) (Figure 4.16(a)). Bacterial filamentation is often observed as a response process to various stresses including DNA damage or inhibition of replication (Figure 4.16(b)) (Bi and Lutkenhaus., 1993). Fimbriae, a long thread-like protein polymer, was also observed (Figure 4.16(c)) in the culture with the presence of the CHI film. Generally, the presence of fimbriae is significantly correlated with pathogenicity and the ability to adhere to and colonize (Klemm., 1985). Nonetheless, the cells undergo filamentation can resume the normal cell proliferation when they are introduced into the new environment without stress (Figure 4.16(d)).

From Figure 4.15, the profound antibacterial activity of doxycycline/MSG loaded CHI/PLA/PCL blend film was exhibited. More than twice of reduction in total cell dry weight was observed in the bacterial culture with the presence of this film compared with the control bacterial culture. This was because the doxycycline rapidly released from the film bound reversibly to the small subunit of bacterial ribosome where it interfacial bound with the charged-tRNA acceptor site. Its action on the culture was bacteriostatic (preventing the bacterial growth) rather than killing (bactericidal) (Gottesmann., 1967; Oehleret al., 1997; Anokhina etal., 2004).

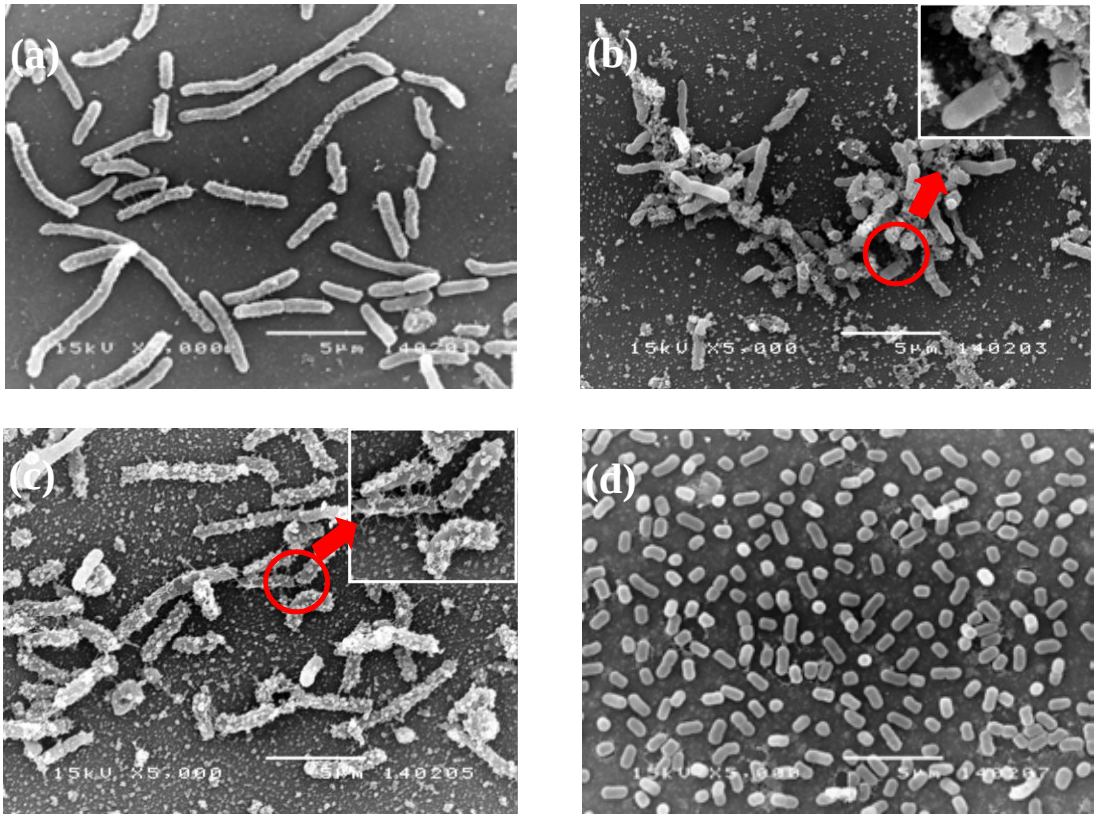


Figure 4.16 Scanning electron micrographs (at the magnification of 5,000) of *E.coli* adhered on the CHI film surface where (a) bacterial filamentation; (b) cell lysis; (c) fimbrae formation; and (d) resumed cell division and growth were observed as the cultivation time proceeded.

CHAPTER V

CONCLUSIONS

5.1 Conclusions

The highest molecular weight Poly(L-lactic acid) of 29,807 was obtained by direct polycondensation of L-lactic acid at 72 hours using $Sb(OAc)_2$ as a catalyst and diphenylether as solvent. Moreover, DSC results showed that poly(L-lactic acid) provided T_g at 55.6°C, and T_m at 146.4°C.

Hemostatic CHI/PLA/PCL wound dressing was prepared by the modified spontaneous emulsification-solvent diffusion method. It was found that the blend film containing 60% CHI, 28% PLA, and 12% with glycerin as emulsifier is suitable for film preparation. The results in this study revealed the possibility of using doxycycline/MSG loaded CHI/PLA/PCL blend film as the hemostatic wound dressing device. From the in vitro study, doxycycline/MSG loaded CHI/PLA/PCL blend film was found to be effectively used to prevent hemolysis and bacterial infection. MSG was proven to be the active ingredient for accelerating blood coagulation while doxycycline provided the antibacterial activity to the blend film. It was found that the CHI/PLA/PCL blend film rapidly and almost completely released MSG and doxycycline loaded onto the film. Released MSG accelerated the blood coagulation and also induced the fibrin formation while the cationic nature of CHI in CHI/PLA/PCL blend film interacted with the negatively charged biological molecules on the red blood cell and platelets surface. In vitro antibacterial activity test revealed that the CHI film caused change of cell proliferation and delayed growth. This phenomenon was more profound in *E. coli* culture compared with *S. aureus*. Cell filamentation and fimbrial formation were clearly seen from the scanning electron micrographs of *E. coli* colonized on the CHI film surface.

5.2 Suggestion for future work

Metal catalyst in polycondensation polymerization of PLLA must be remove from the synthesized PLLA and should be confirmed with atomic absorption spectroscopy (AAS).

For further study, wound dressing should be prepared using different methods including electrospinning, polymer nanoparticles or film forming solution to improve mechanical property and controlled drug release. With further *in vivo* study on tested animals and clinical trial, it was expected that this device could be of commercial interest.

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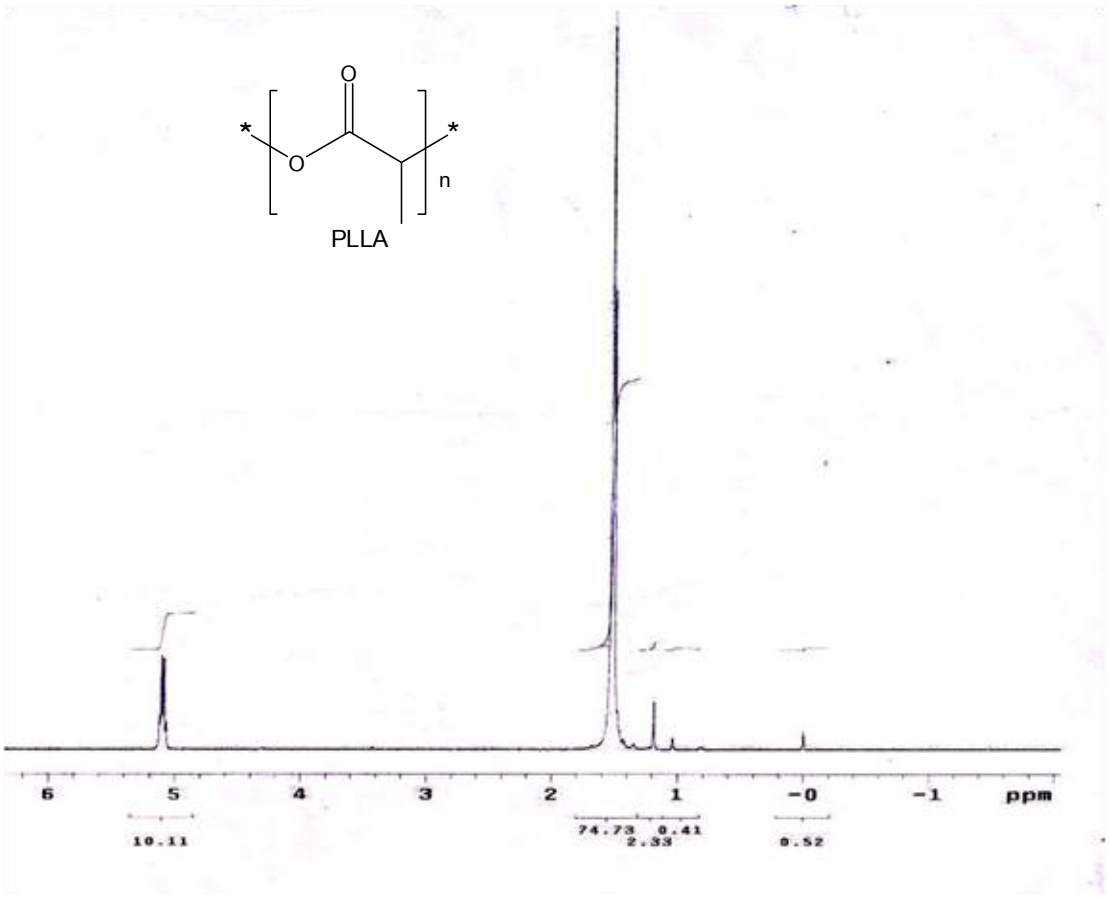
APPENDICES

APPENDIX A

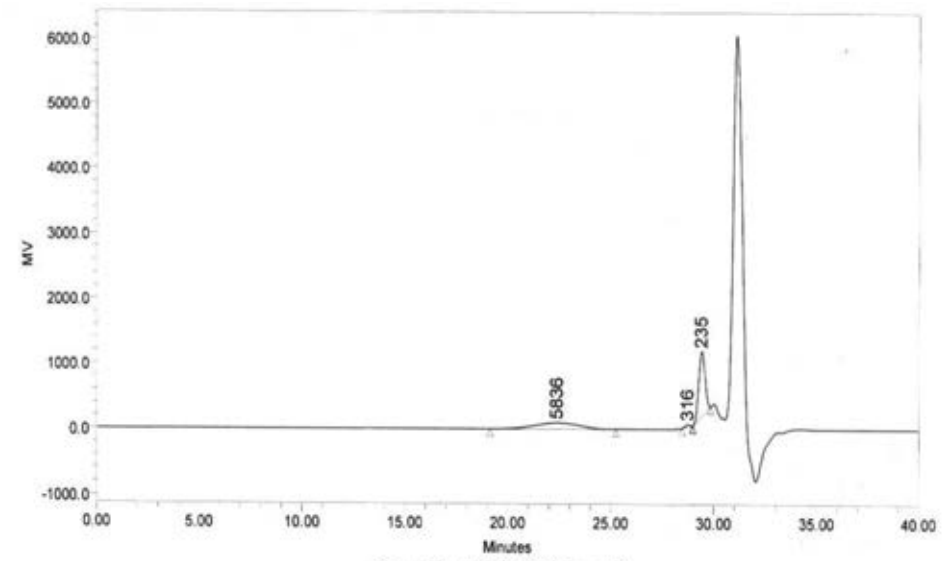
^{1}H NMR SPECTRUM, GPC AND DSC CHROMATOGRAM



^{1}H NMR spectrum

Figure A1 ^{1}H NMR spectrum of PLLA obtained by direct polycondensation at 140°C with $Sb(OAc)_2$

GPC chromatogram

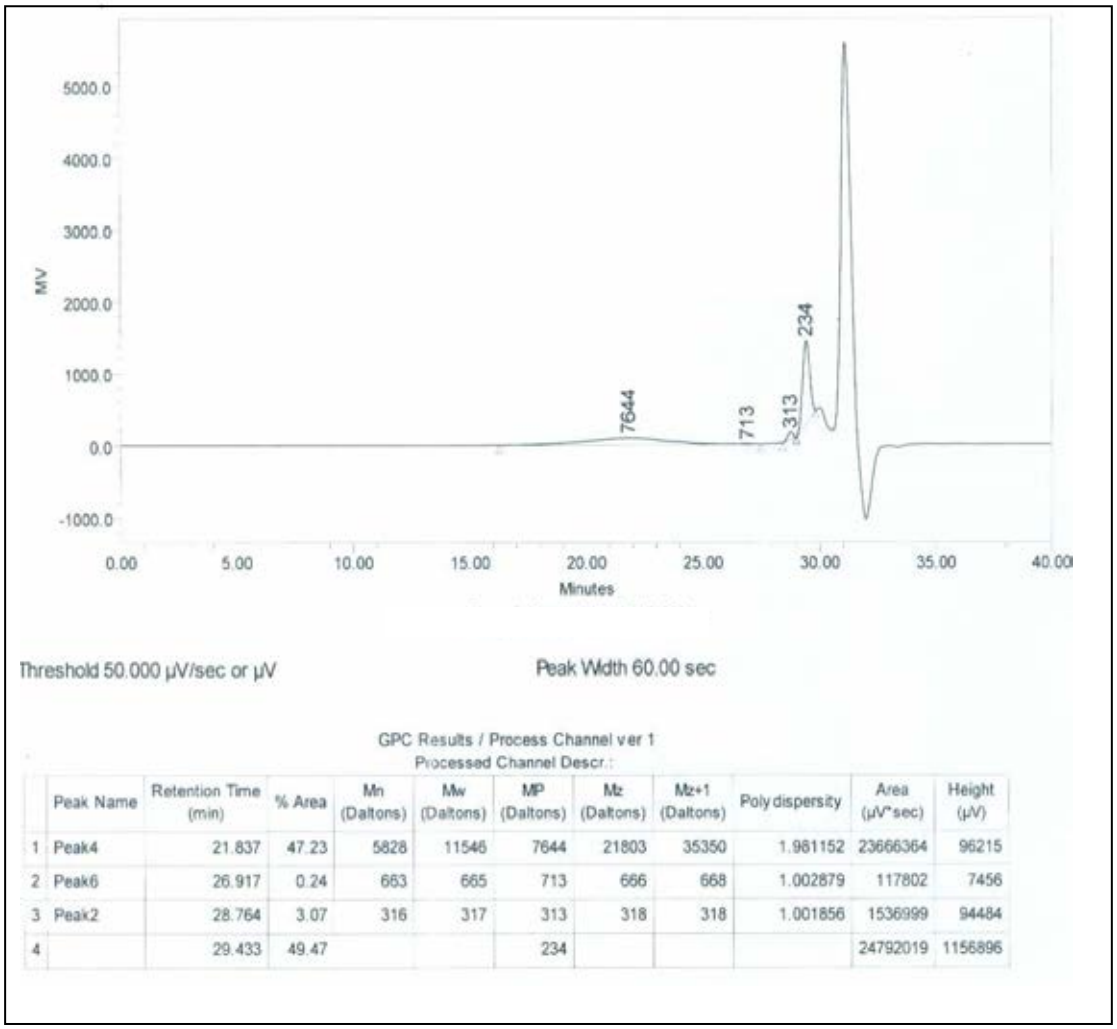


Threshold 50.000 µV/sec or µV Peak Width 60.00 sec

GPC Results / Process Channel ver 1
Processed Channel Descr.:

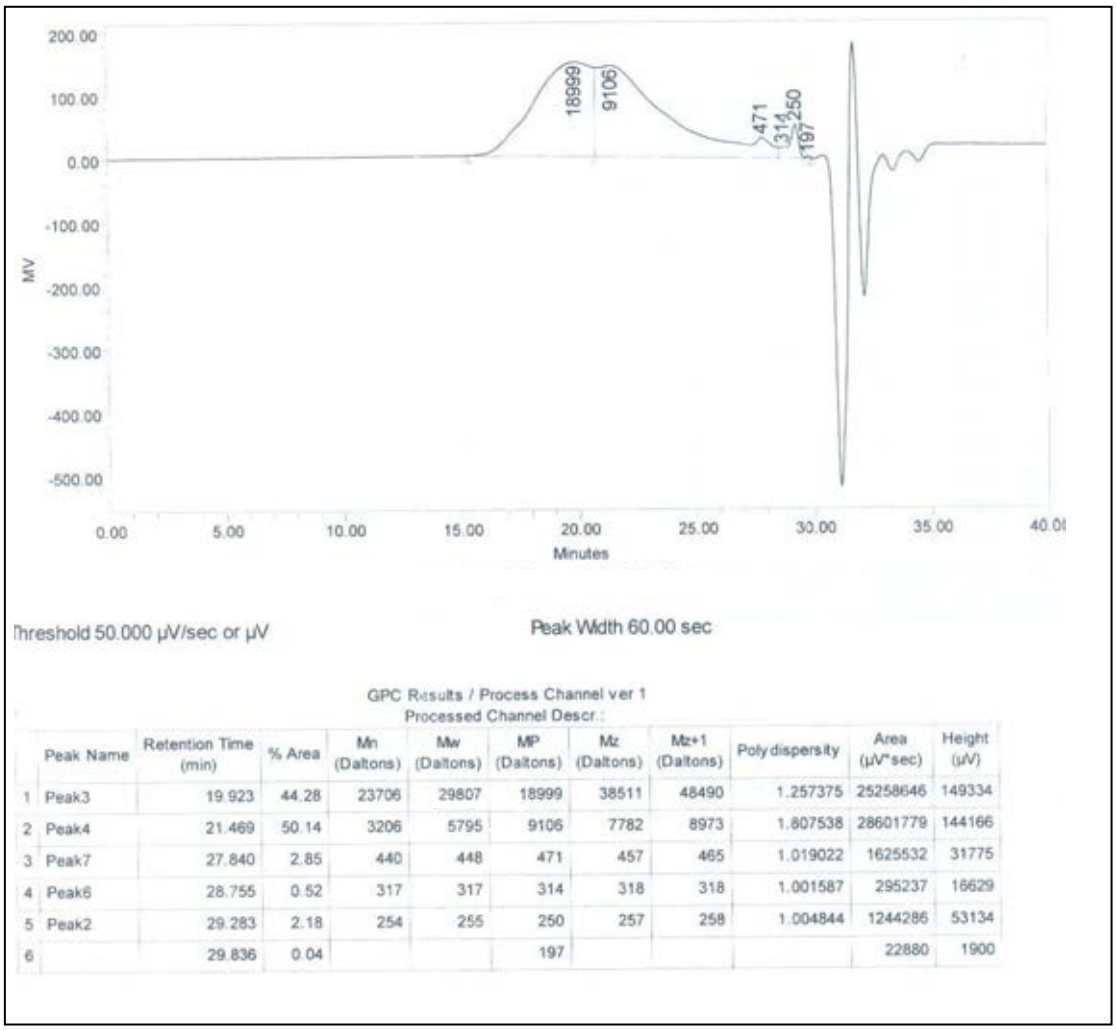
	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak4	22.406	39.33	5452	6690	5836	8270	10123	1.227240	14296914	95054
2	Peak2	28.741	1.83	320	320	316	321	321	1.001657	665107	42890
3	Peak7	29.421	58.85	235	236	235	236	237	1.003470	21393609	997285

Figure A2 GPC chromatogram of PLLA obtained by direct polycondensation at 140°C with $Sb(OAc)_2$ for 24 h.



	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak4	21.837	47.23	5828	11546	7644	21803	35350	1.981152	23666364	96215
2	Peak6	26.917	0.24	663	665	713	666	668	1.002879	117802	7456
3	Peak2	28.764	3.07	316	317	313	318	318	1.001856	1536999	94484
4		29.433	49.47			234				24792019	1156896

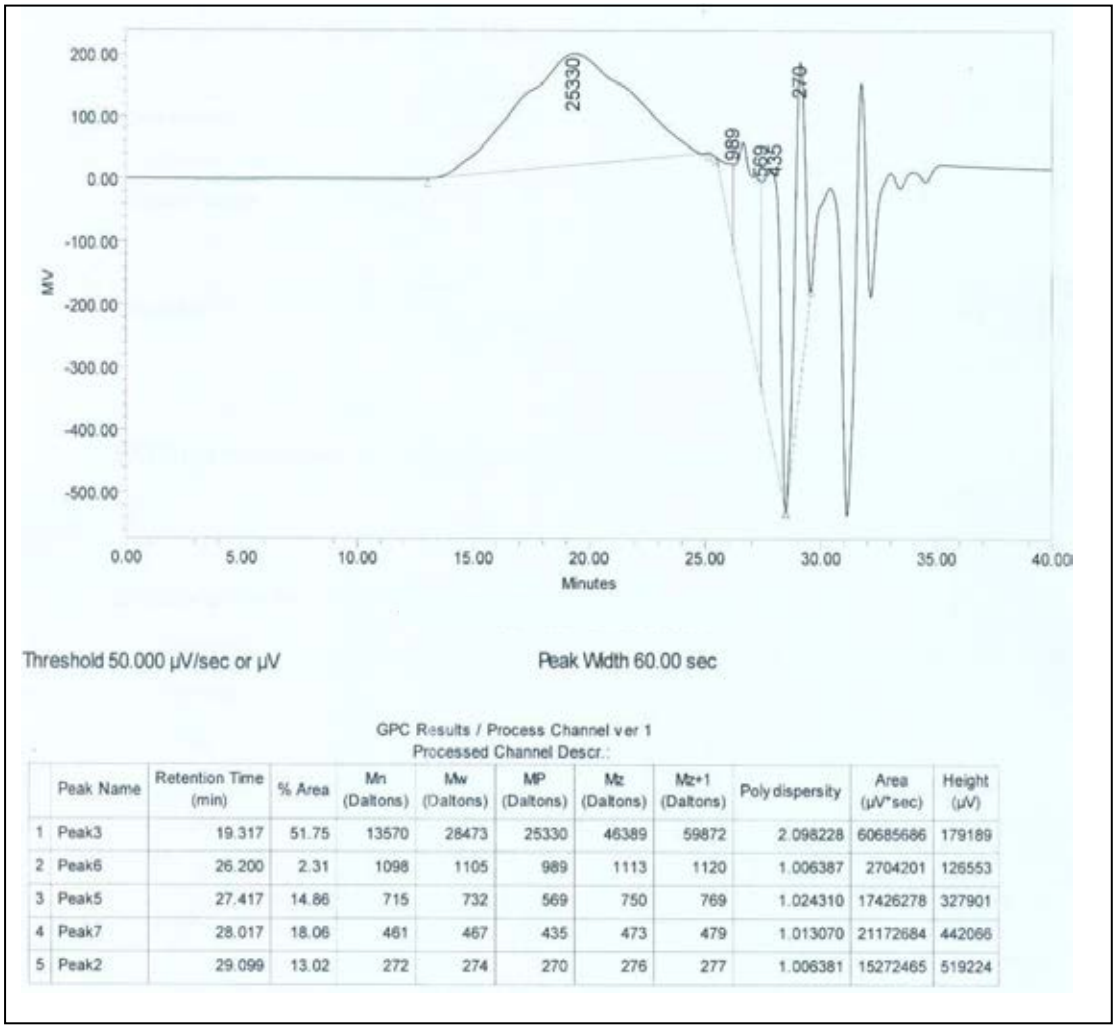
Figure A3 GPC chromatogram of PLLA obtained by direct polycondensation at 140°C with $Sb(OAc)_2$ for 48 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak3	19.923	44.28	23706	29807	18999	38511	48490	1.257375	25258646	149334
2	Peak4	21.469	50.14	3206	5795	9106	7782	8973	1.807538	28601779	144166
3	Peak7	27.840	2.85	440	448	471	457	465	1.019022	1625532	31775
4	Peak6	28.755	0.52	317	317	314	318	318	1.001587	295237	16629
5	Peak2	29.283	2.18	254	255	250	257	258	1.004844	1244286	53134
6		29.836	0.04			197				22880	1900

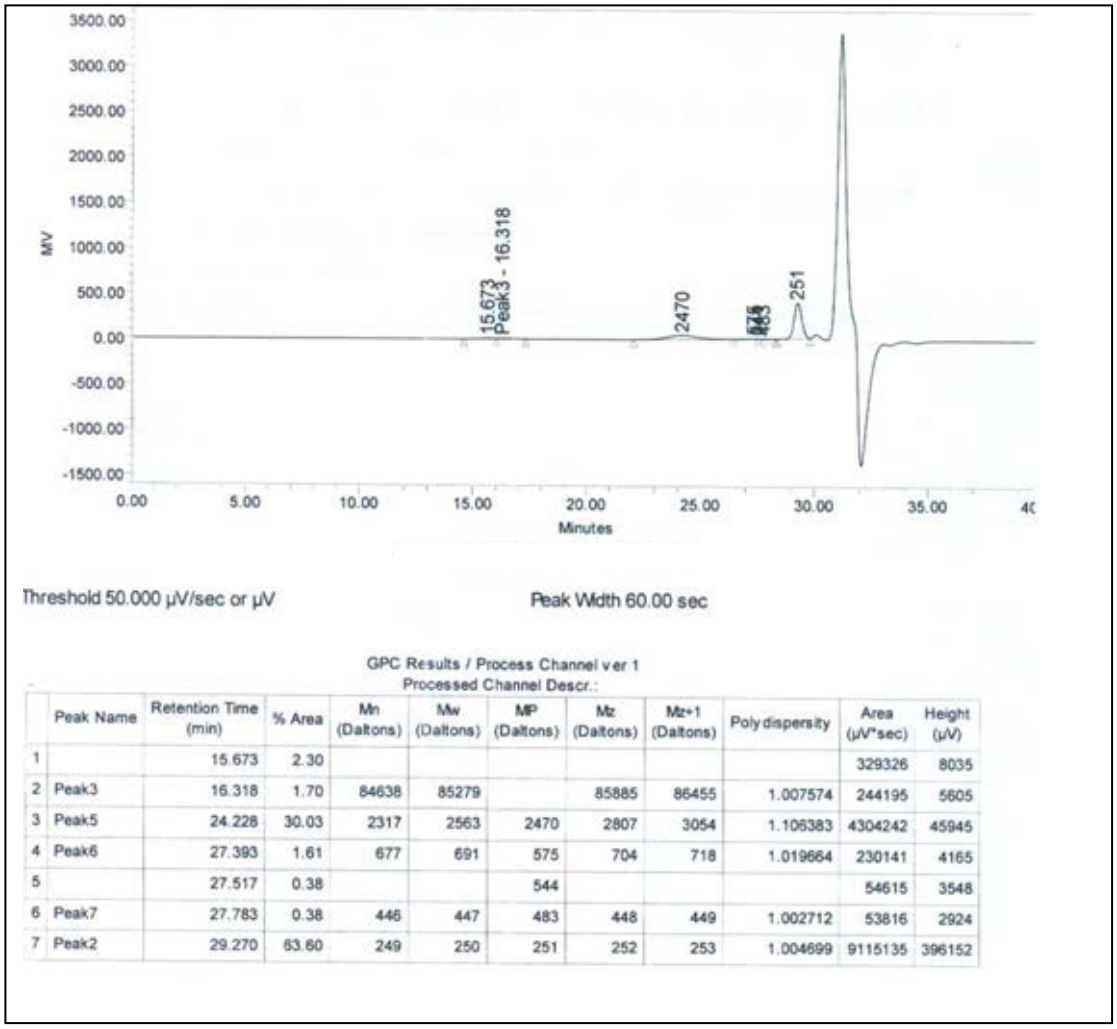
Figure A4 GPC chromatogram of PLLA obtained by direct polycondensation at 140°C with $Sb(OAc)_2$ for 72 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak3	19.317	51.75	13570	28473	25330	46389	59872	2.098228	60685686	179189
2	Peak6	26.200	2.31	1098	1105	989	1113	1120	1.006387	2704201	126553
3	Peak5	27.417	14.86	715	732	569	750	769	1.024310	17426278	327901
4	Peak7	28.017	18.06	461	467	435	473	479	1.013070	21172684	442066
5	Peak2	29.099	13.02	272	274	270	276	277	1.006381	15272465	519224

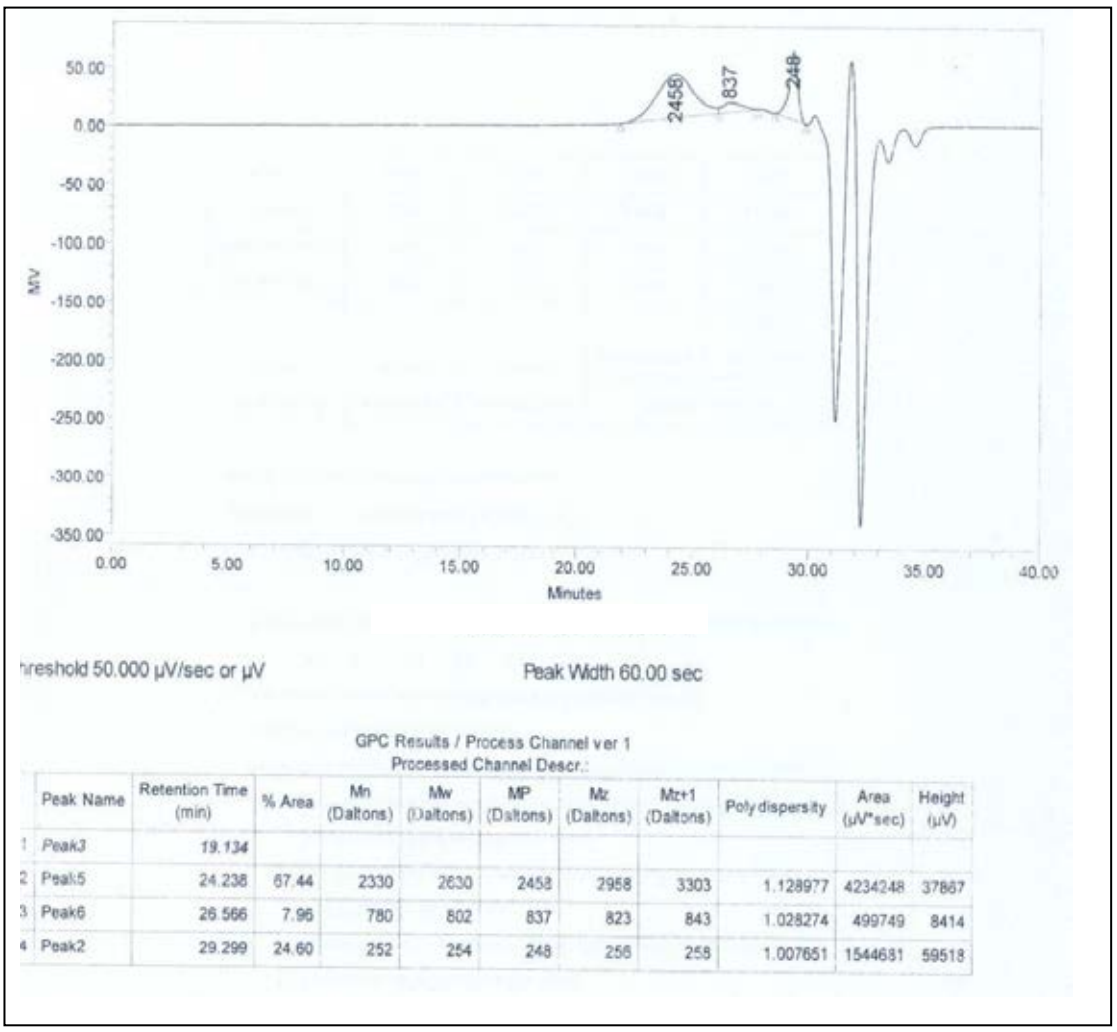
Figure A5 GPC chromatogram of PLLA obtained by direct polycondensation at 140°C with $Sb(OAc)_2$ for 96 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1		15.673	2.30							329326	8035
2	Peak3	16.318	1.70	84638	85279		85885	86455	1.007574	244195	5605
3	Peak5	24.228	30.03	2317	2563	2470	2807	3054	1.106383	4304242	45945
4	Peak6	27.393	1.61	677	691	575	704	718	1.019664	230141	4165
5		27.517	0.38			544				54615	3548
6	Peak7	27.783	0.38	446	447	483	448	449	1.002712	53816	2924
7	Peak2	29.270	63.60	249	250	251	252	253	1.004699	9115135	396152

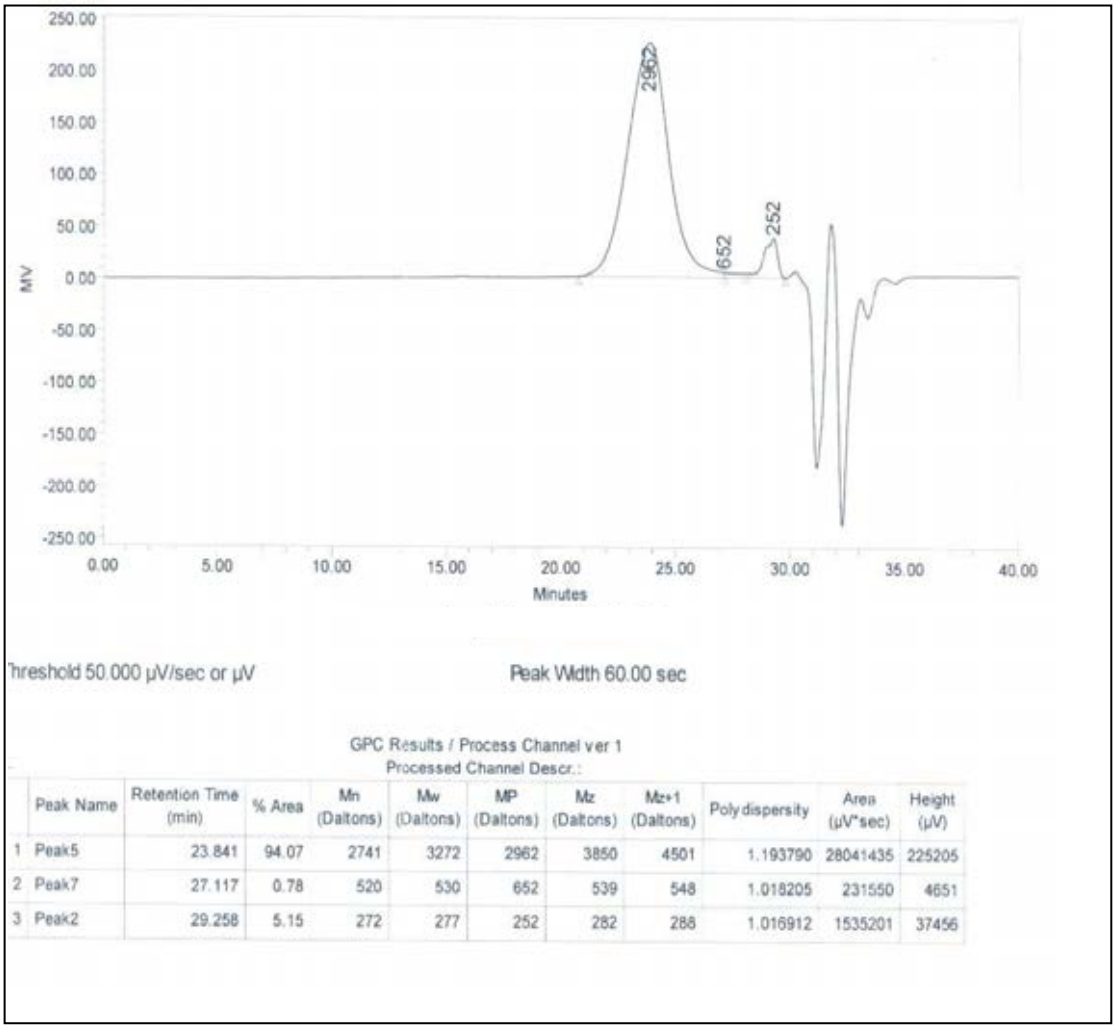
Figure A6 GPC chromatogram of PLLA obtained by direct polycondensation at 140°C with $Co(OAc)_2$ for 24 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak3	19.134									
2	Peak5	24.238	67.44	2330	2630	2458	2958	3303	1.128977	4234248	37867
3	Peak6	26.566	7.96	780	802	837	823	843	1.028274	499749	8414
4	Peak2	29.299	24.60	252	254	248	256	258	1.007651	1544681	59518

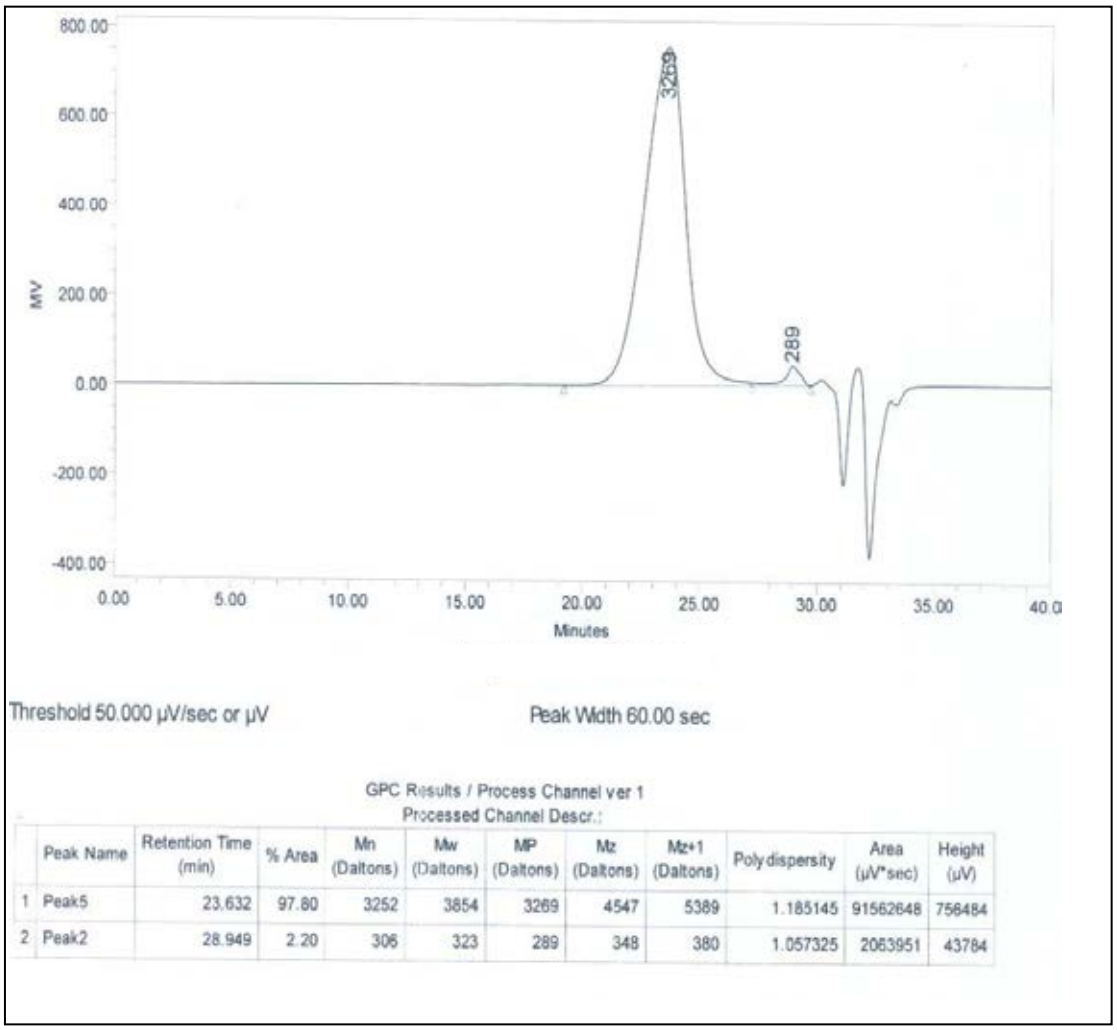
Figure A7 GPC chromatogram of PLLA obtained by direct polycondensation at 140°C with $Co(OAc)_2$ for 48 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

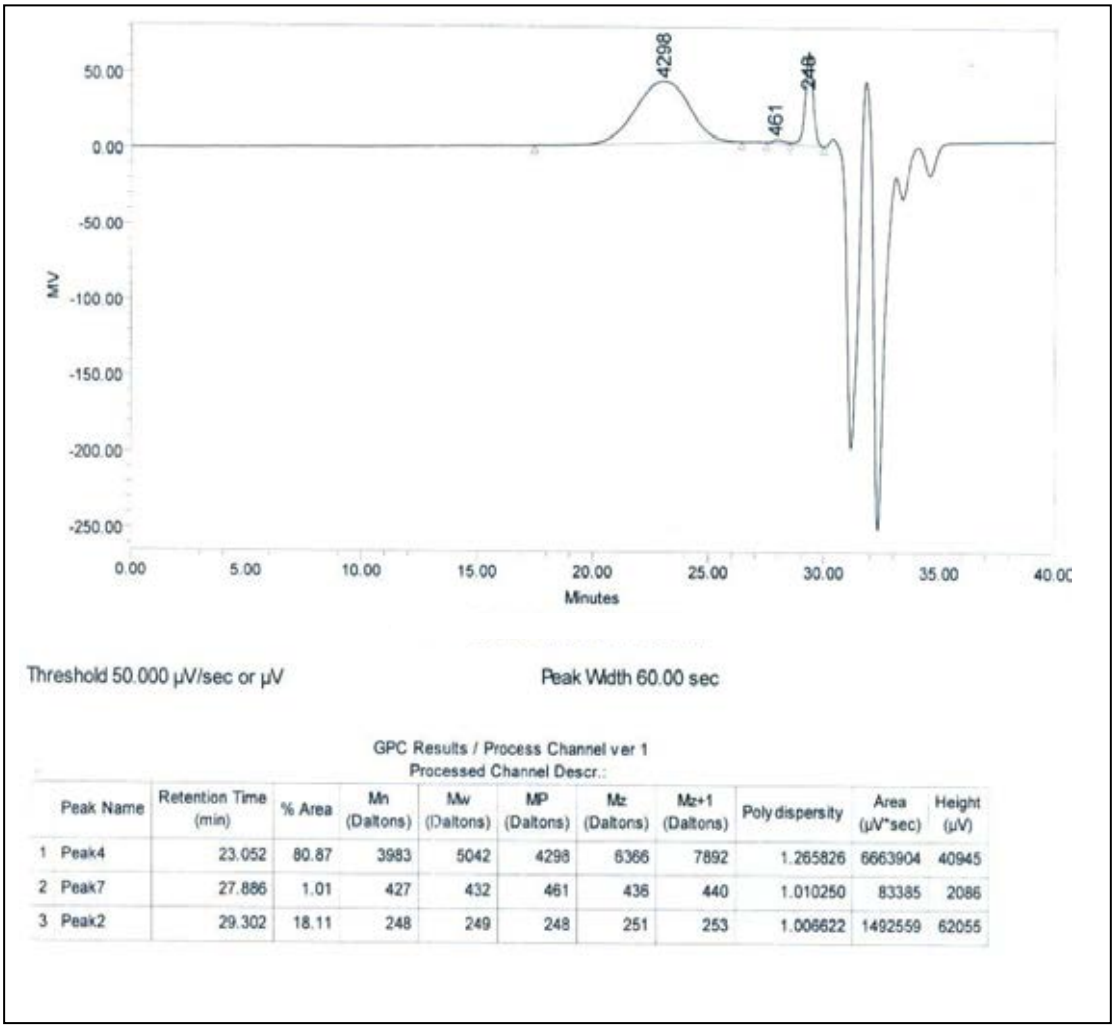
	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Area (µV*sec)	Height (µV)
1	Peak5	23.841	94.07	2741	3272	2962	3850	4501	1.193790	28041435	225205
2	Peak7	27.117	0.78	520	530	652	539	548	1.018205	231550	4651
3	Peak2	29.258	5.15	272	277	252	282	288	1.016912	1535201	37456

Figure A8 GPC chromatogram of PLLA obtained by direct polycondensation at 140^{o}C with $Co(OAc)_2$ for 72 h.



	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Area (µV*sec)	Height (µV)
1	Peak5	23.632	97.80	3252	3854	3269	4547	5389	1.185145	91562648	756484
2	Peak2	28.949	2.20	306	323	289	348	380	1.057325	2063951	43784

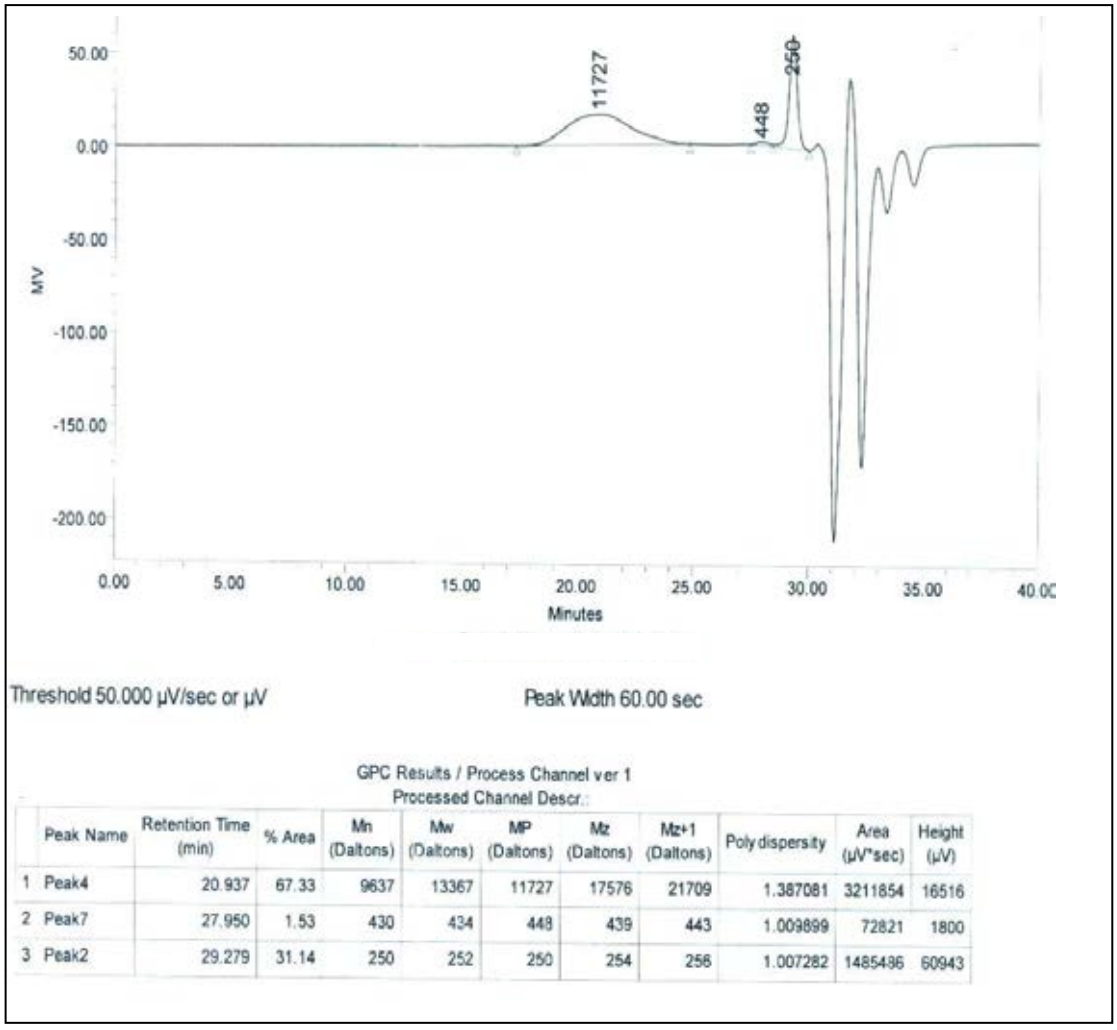
Figure A9 GPC chromatogram of PLLA obtained by direct polycondensation at 140°C with $Co(OAc)_2$ for 96 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak4	23.052	80.87	3983	5042	4298	6366	7892	1.265826	6663904	40945
2	Peak7	27.886	1.01	427	432	461	436	440	1.010250	83385	2086
3	Peak2	29.302	18.11	248	249	248	251	253	1.006622	1492559	62055

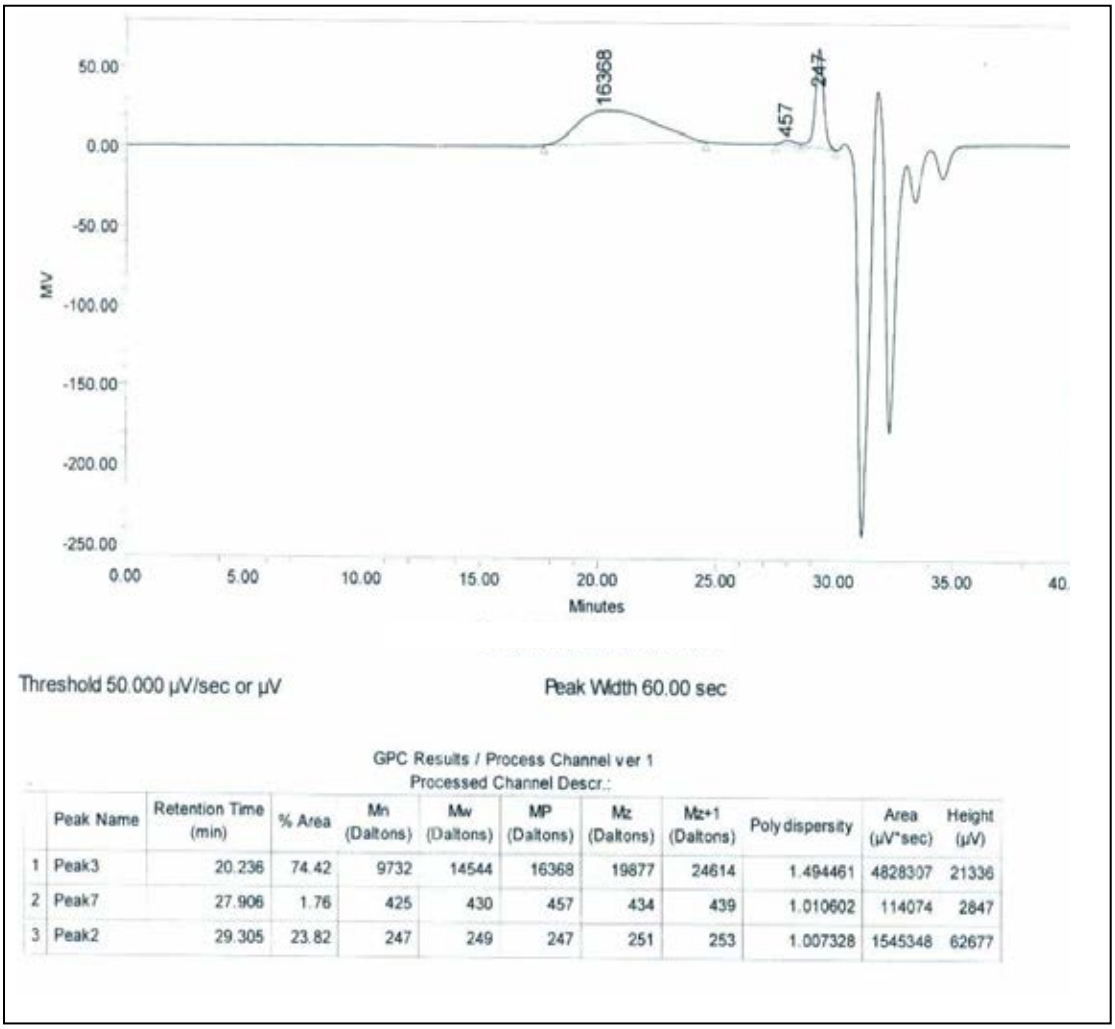
Figure A10 GPC chromatogram of PLLA obtained by direct polycondensation at 140^{o}C with $Sn(Oct)_2$ for 24 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Area (µV*sec)	Height (µV)
1	Peak4	20.937	67.33	9637	13367	11727	17576	21709	1.387081	3211854	16516
2	Peak7	27.950	1.53	430	434	448	439	443	1.009899	72821	1800
3	Peak2	29.279	31.14	250	252	250	254	256	1.007282	1485486	60943

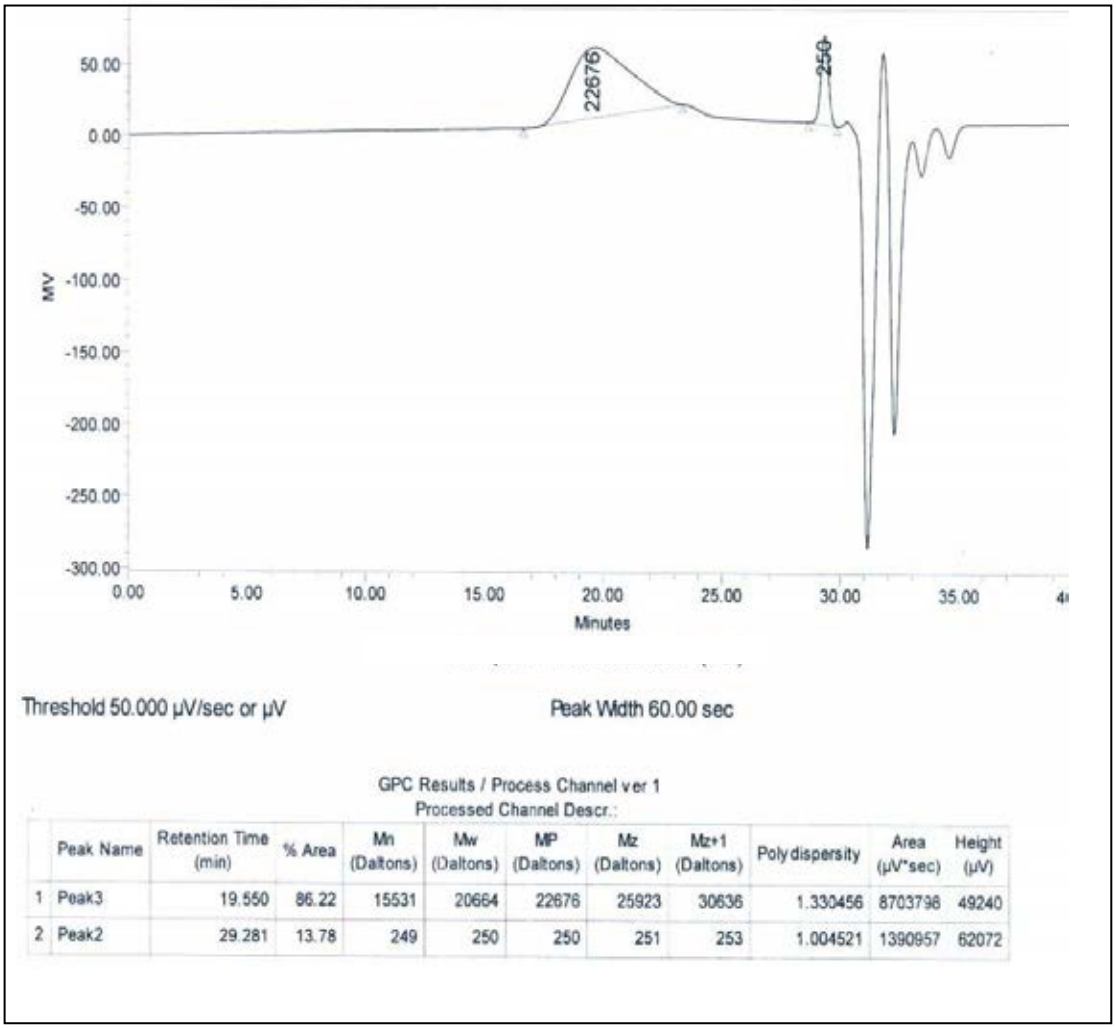
Figure A11 GPC chromatogram of PLLA obtained by direct polycondensation at 140°C with $Sn(Oct)_2$ for 48 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak3	20.236	74.42	9732	14544	16368	19877	24614	1.494461	4828307	21336
2	Peak7	27.906	1.76	425	430	457	434	439	1.010602	114074	2847
3	Peak2	29.305	23.82	247	249	247	251	253	1.007328	1545348	62677

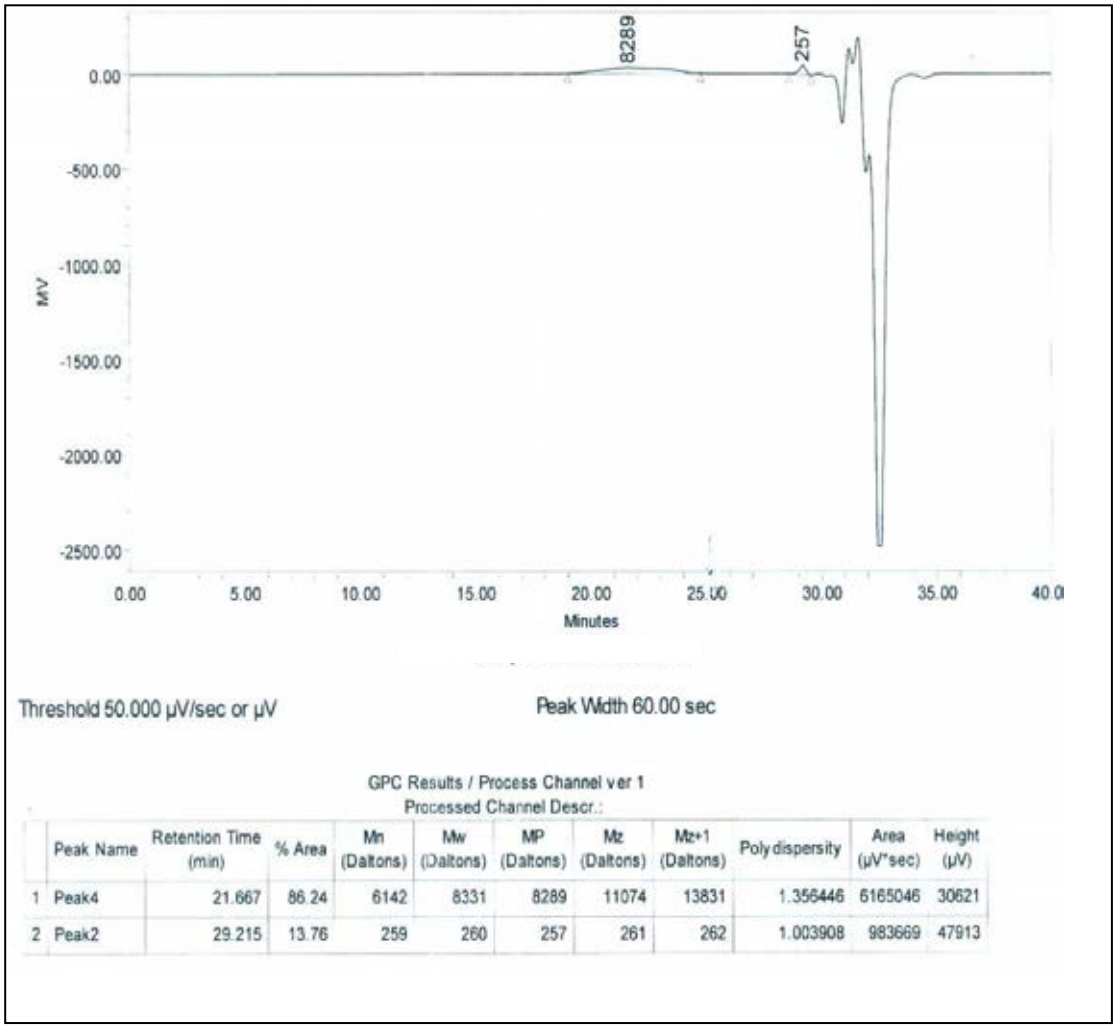
Figure A12 GPC chromatogram of PLLA obtained by direct polycondensation at 140^{o}C with $Sn(Oct)_2$ for 72 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Area (µV*sec)	Height (µV)
1	Peak3	19.550	86.22	15531	20664	22676	25923	30636	1.330456	8703798	49240
2	Peak2	29.281	13.78	249	250	250	251	253	1.004521	1390957	62072

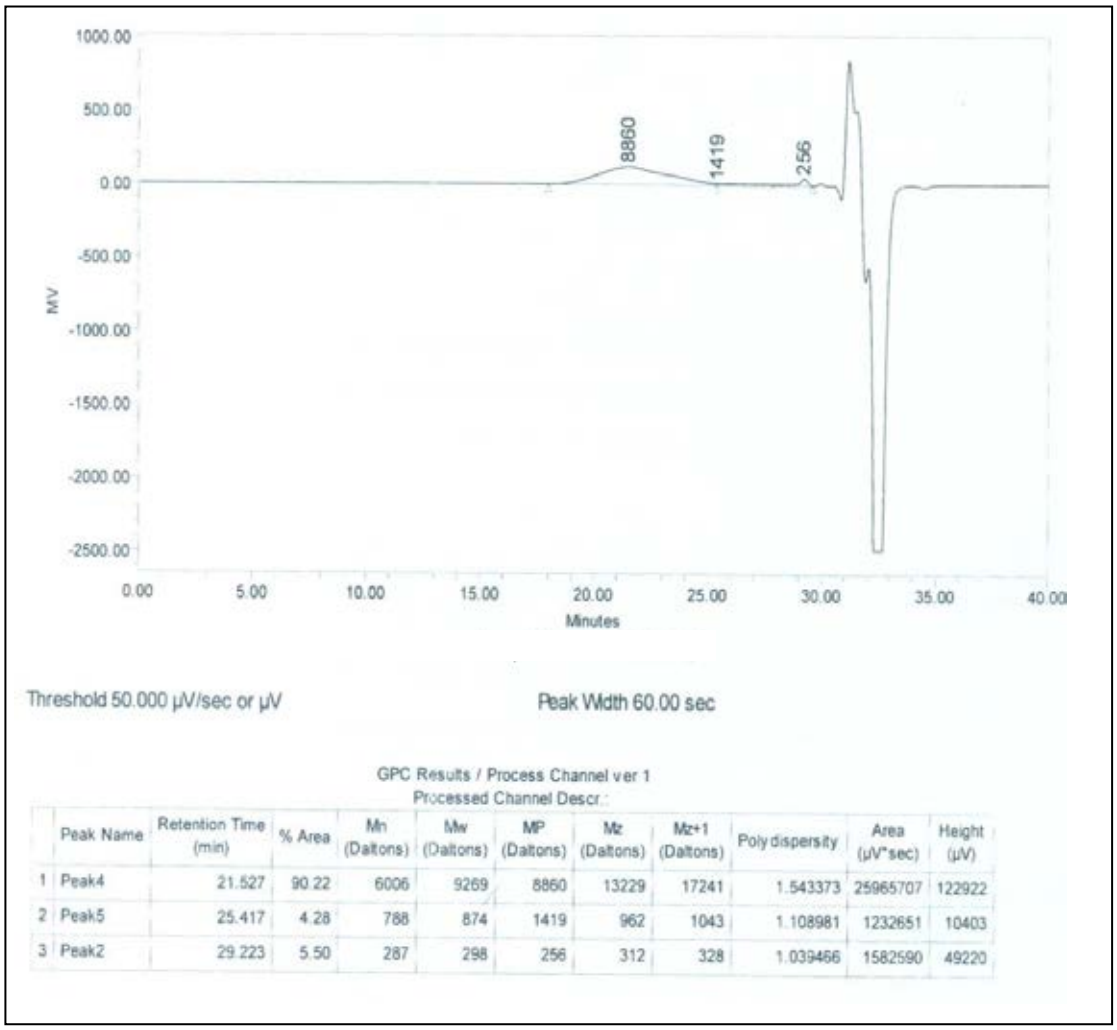
Figure A13 GPC chromatogram of PLLA obtained by direct polycondensation at 140°C with $Sn(Oct)_2$ for 96 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

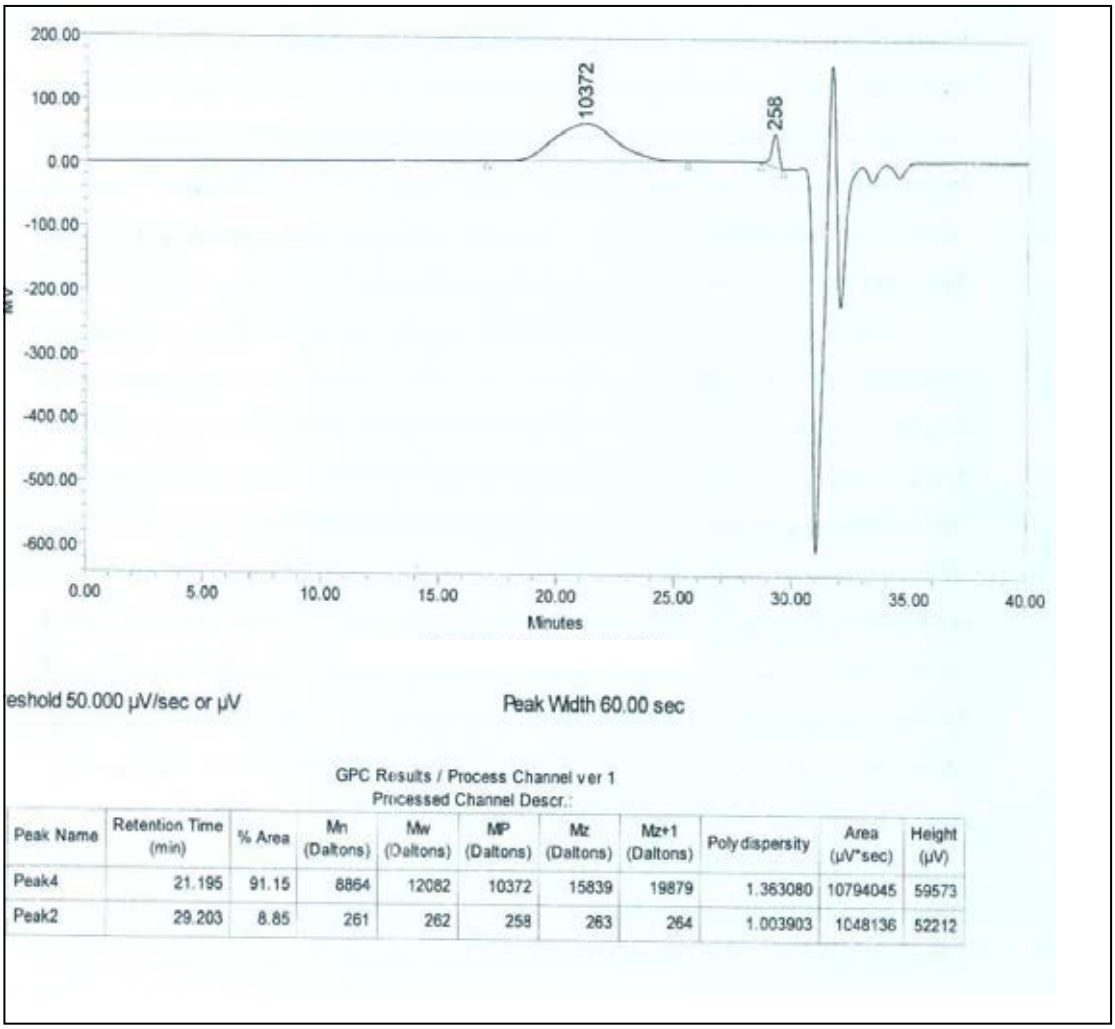
	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak4	21.667	86.24	6142	8331	8289	11074	13831	1.356446	6165046	30621
2	Peak2	29.215	13.76	259	260	257	261	262	1.003908	983669	47913

Figure A14 GPC chromatogram of PLLA obtained by direct polycondensation at 160°C with $Sb(OAc)_2$ for 24 h.



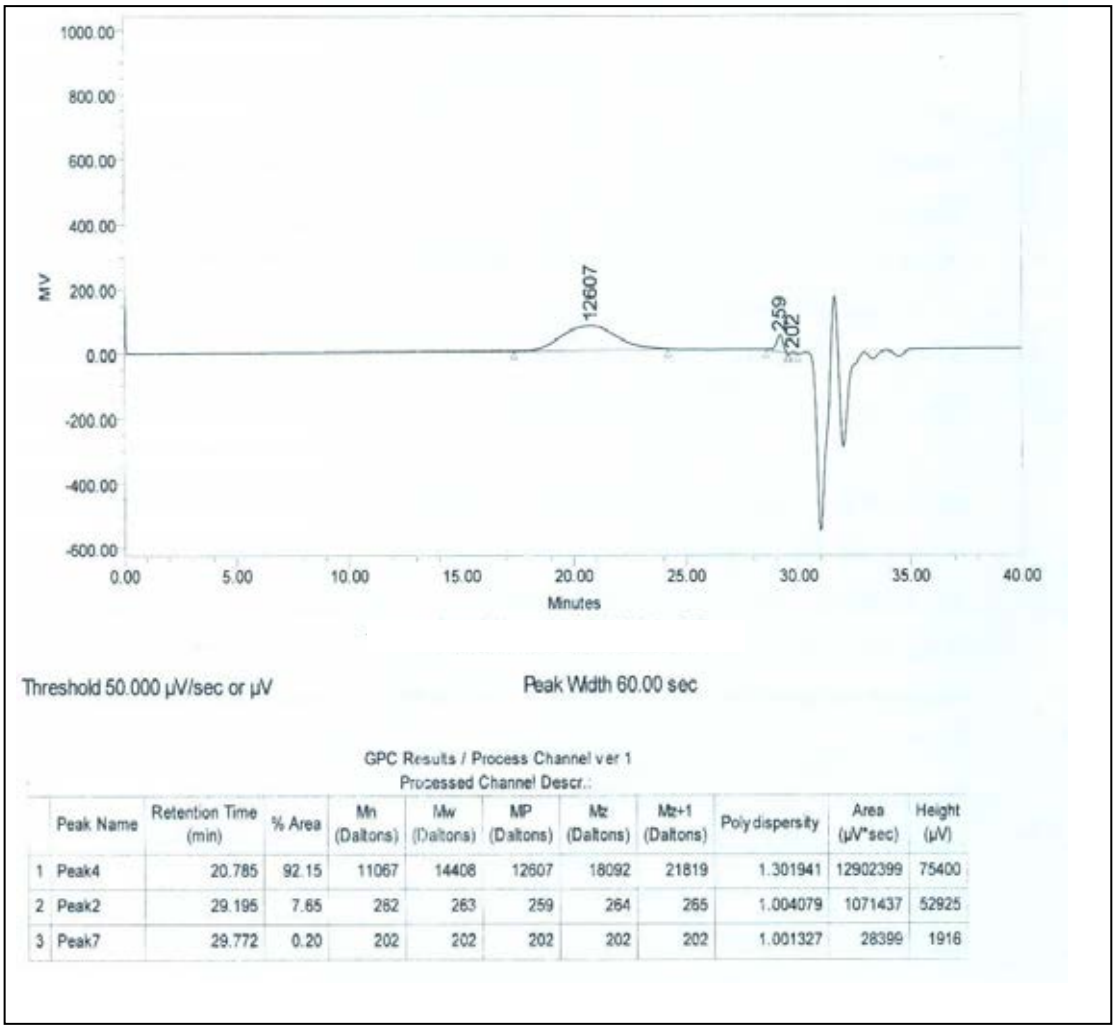
	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak4	21.527	90.22	6006	9269	8860	13229	17241	1.543373	25965707	122922
2	Peak5	25.417	4.28	788	874	1419	962	1043	1.108981	1232651	10403
3	Peak2	29.223	5.50	287	298	256	312	328	1.039466	1582590	49220

Figure A15 GPC chromatogram of PLLA obtained by direct polycondensation at 160^{o}C with $Sb(OAc)_2$ for 48 h.



Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Area (µV*sec)	Height (µV)
Peak4	21.195	91.15	8864	12082	10372	15839	19879	1.363080	10794045	59573
Peak2	29.203	8.85	261	262	258	263	264	1.003903	1048136	52212

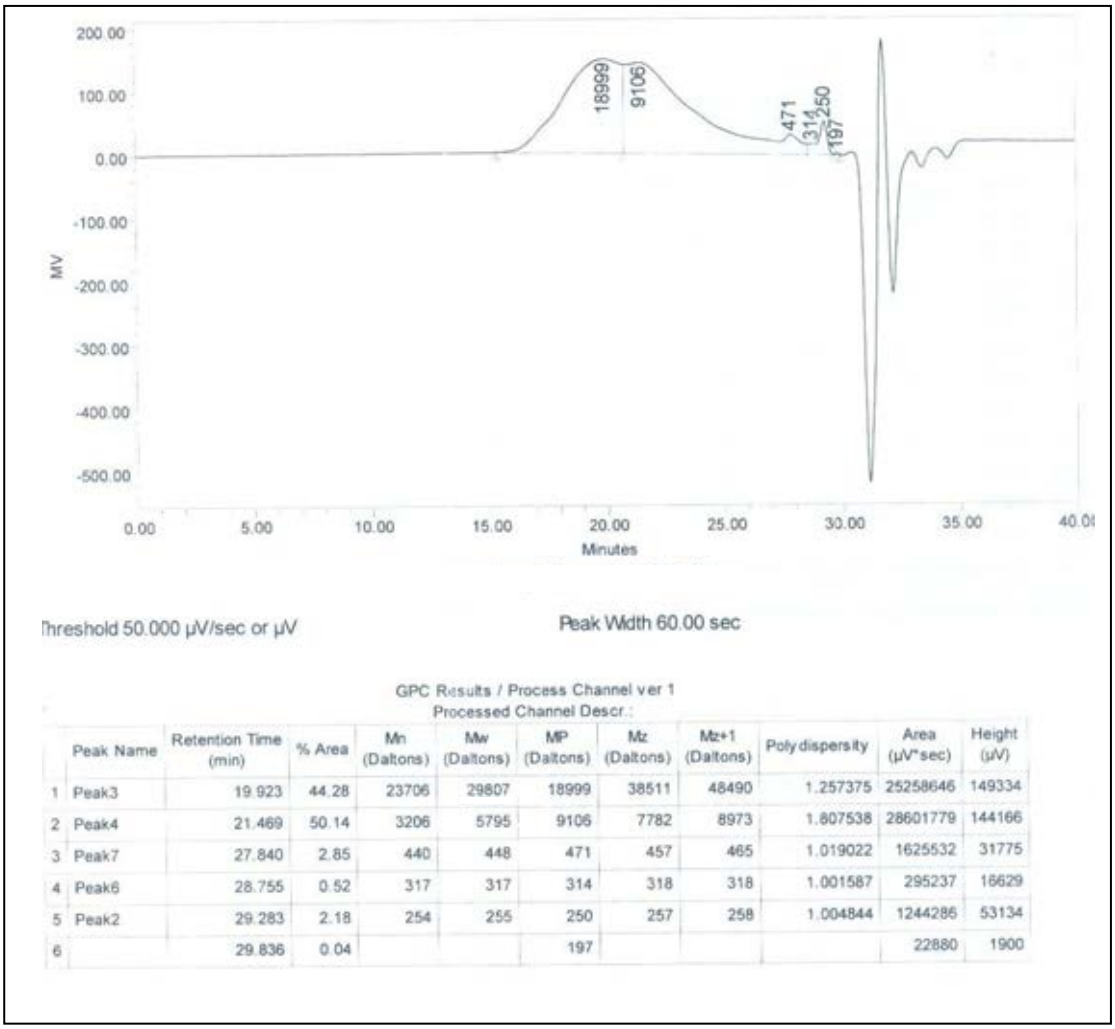
Figure A16 GPC chromatogram of PLLA obtained by direct polycondensation at 160°C with $Sb(OAc)_2$ for 72 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Area (µV*sec)	Height (µV)
1	Peak4	20.785	92.15	11067	14408	12607	18092	21819	1.301941	12902399	75400
2	Peak2	29.195	7.65	262	263	259	264	265	1.004079	1071437	52925
3	Peak7	29.772	0.20	202	202	202	202	202	1.001327	28399	1916

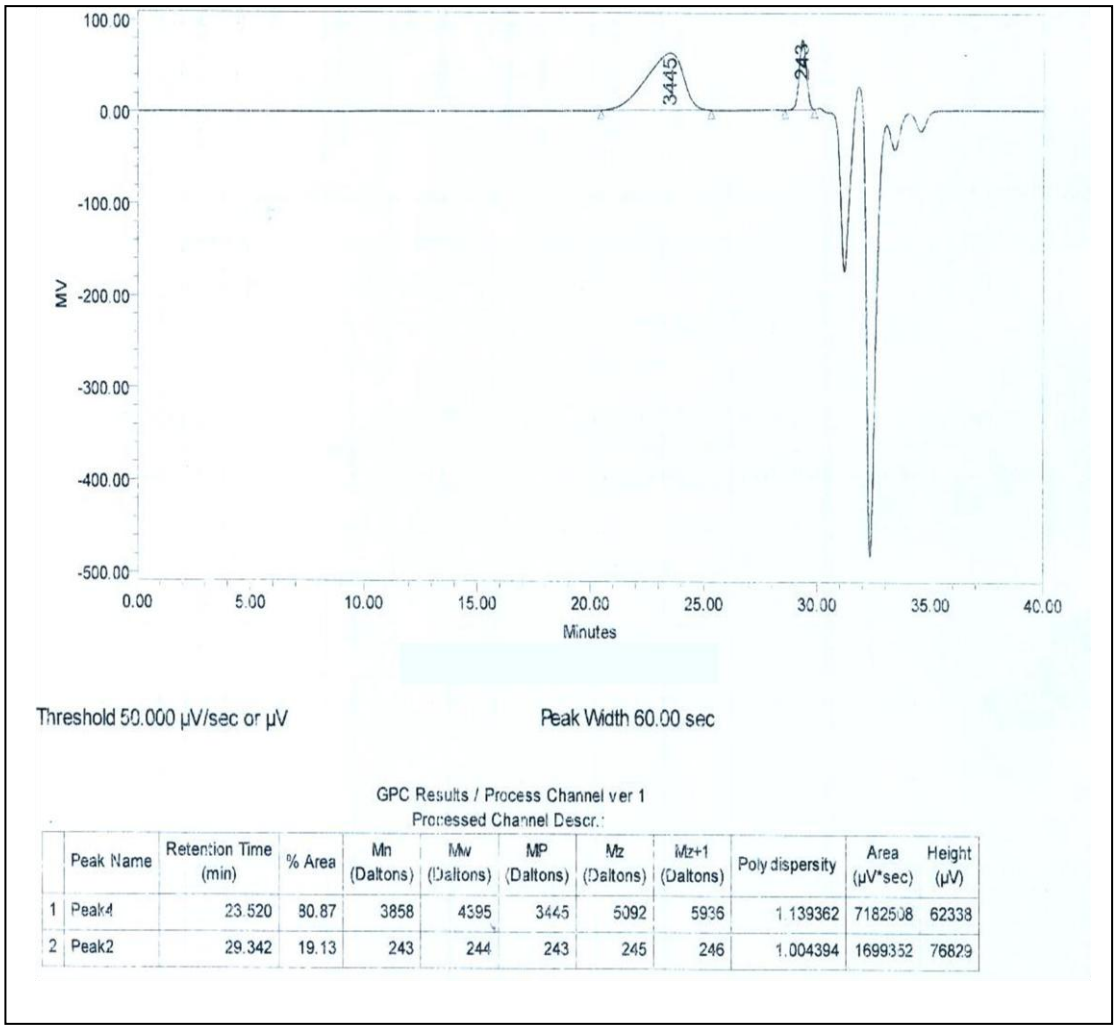
Figure A17 GPC chromatogram of PLLA obtained by direct polycondensation at 160^{o}C with $Sb(OAc)_2$ for 96 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Polydispersity	Area (µV*sec)	Height (µV)
1	Peak3	19.923	44.28	23706	29807	18999	38511	48490	1.257375	25258646	149334
2	Peak4	21.469	50.14	3206	5795	9106	7782	8973	1.807538	28601779	144166
3	Peak7	27.840	2.85	440	448	471	457	465	1.019022	1625532	31775
4	Peak6	28.755	0.52	317	317	314	318	318	1.001587	295237	16629
5	Peak2	29.283	2.18	254	255	250	257	258	1.004844	1244286	53134
6		29.836	0.04			197				22880	1900

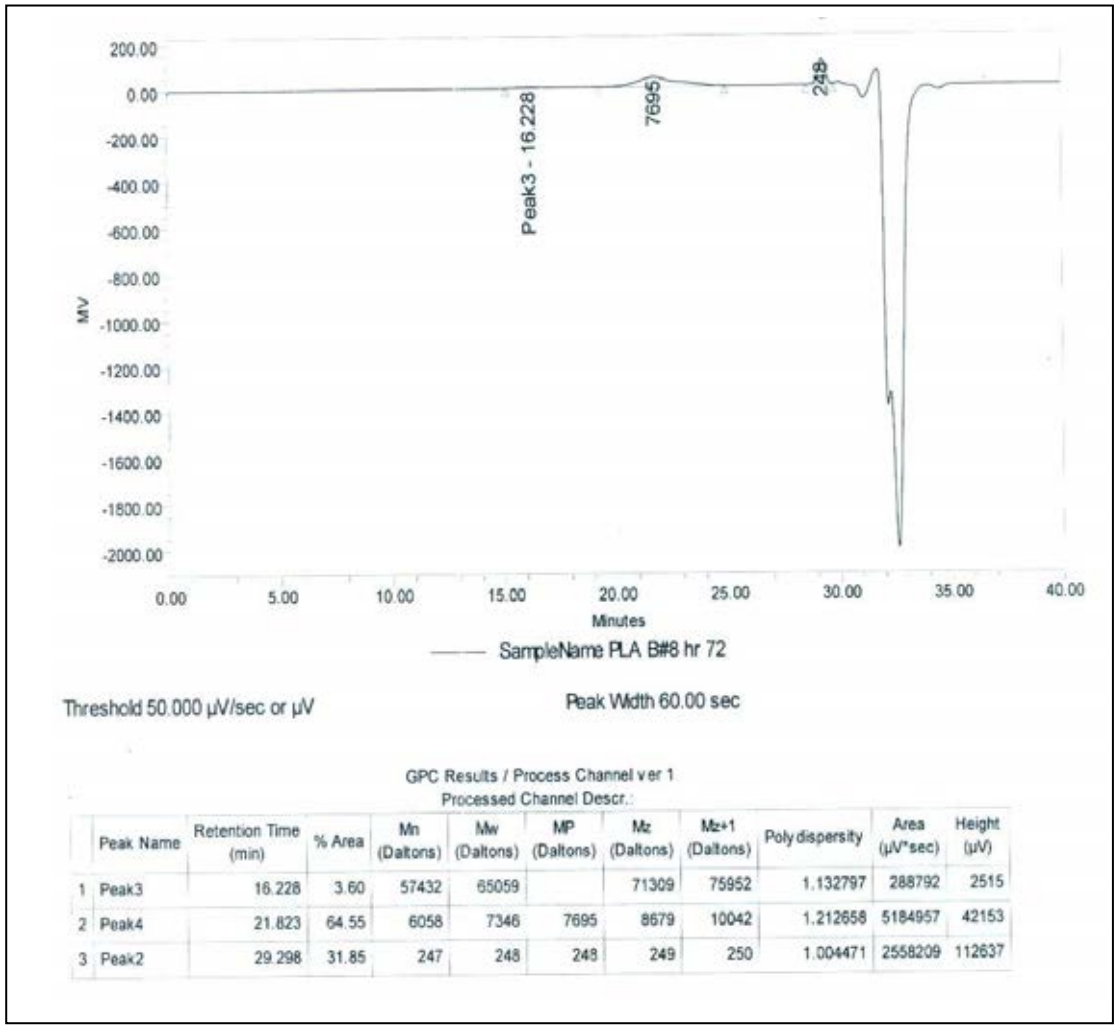
Figure A18 GPC chromatogram of PLLA obtained by direct polycondensation at 200^{o}C with $Sb(OAc)_2$ for 24 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak4	23.520	80.87	3858	4395	3445	5092	5936	1.139362	7182508	62338
2	Peak2	29.342	19.13	243	244	243	245	246	1.004394	1699352	76829

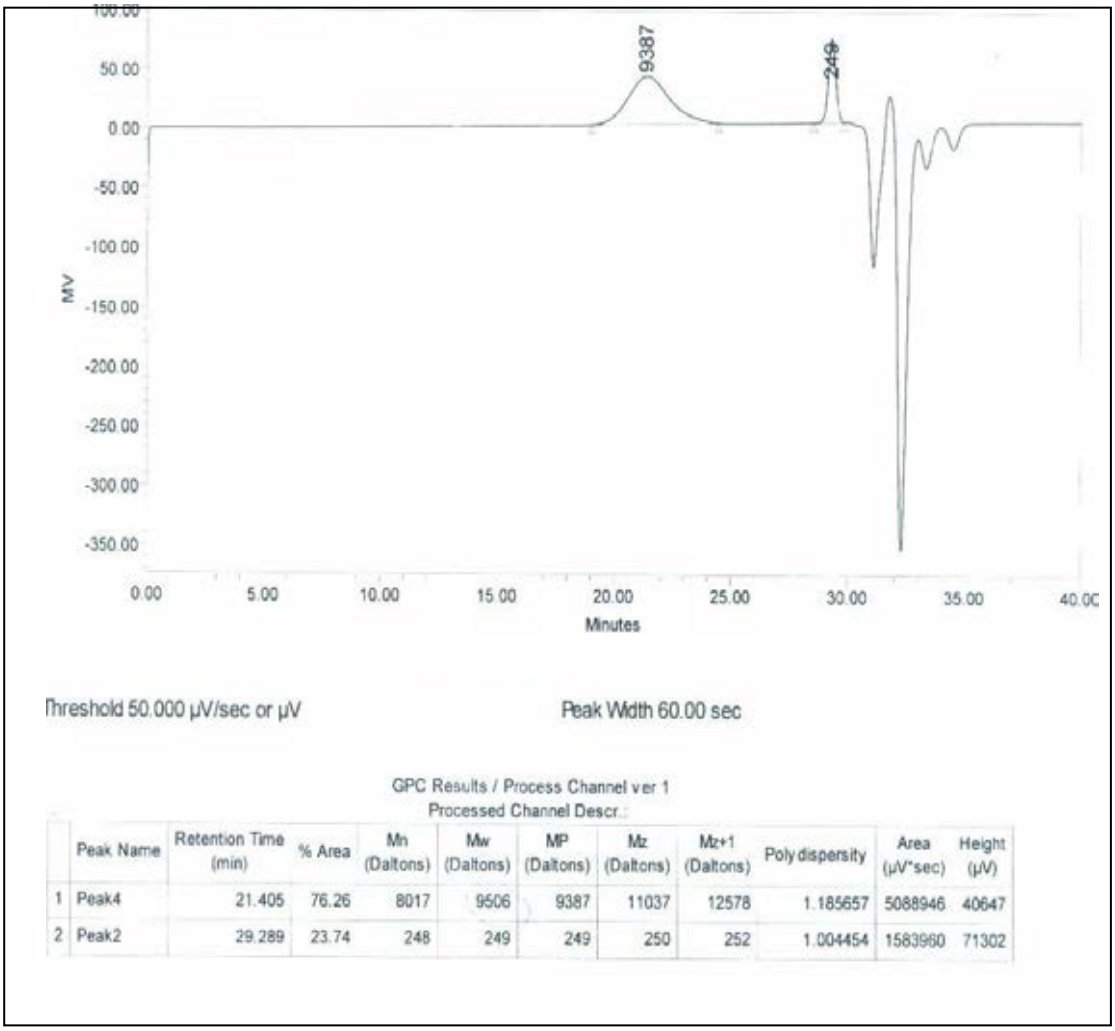
Figure A19 GPC chromatogram of PLLA obtained by direct polycondensation at 200°C with $Sb(OAc)_2$ for 24 h.



GPC Results / Process Channel ver 1
Processed Channel Descr.:

	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak3	16.228	3.60	57432	65059		71309	75952	1.132797	288792	2515
2	Peak4	21.823	64.55	6058	7346	7695	8679	10042	1.212658	5184957	42153
3	Peak2	29.298	31.85	247	248	248	249	250	1.004471	2558209	112637

Figure A20 GPC chromatogram of PLLA obtained by direct polycondensation at 200^{o}C with $Sb(OAc)_2$ for 24 h.



	Peak Name	Retention Time (min)	% Area	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz (Daltons)	Mz+1 (Daltons)	Poly dispersity	Area (µV*sec)	Height (µV)
1	Peak4	21.405	76.26	8017	9506	9387	11037	12578	1.185657	5088946	40647
2	Peak2	29.289	23.74	248	249	249	250	252	1.004454	1583960	71302

Figure A21 GPC chromatogram of PLLA obtained by direct polycondensation at 200^{o}C with $Sb(OAc)_2$ for 24 h.

DSC chromatogram

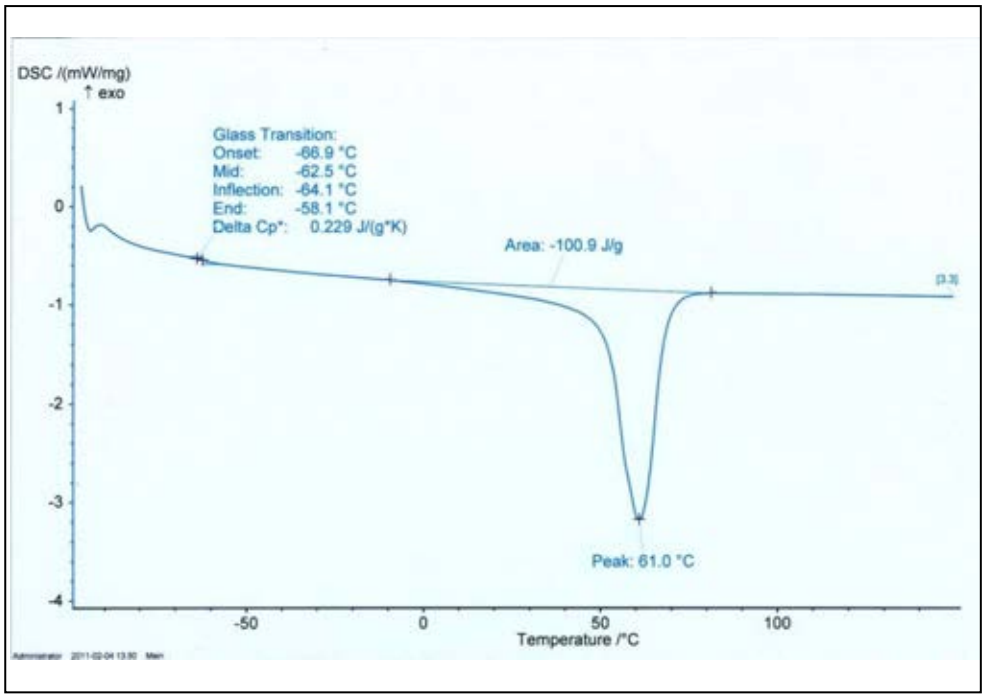


Figure A22 DSC chromatogram of polycaprolactone.

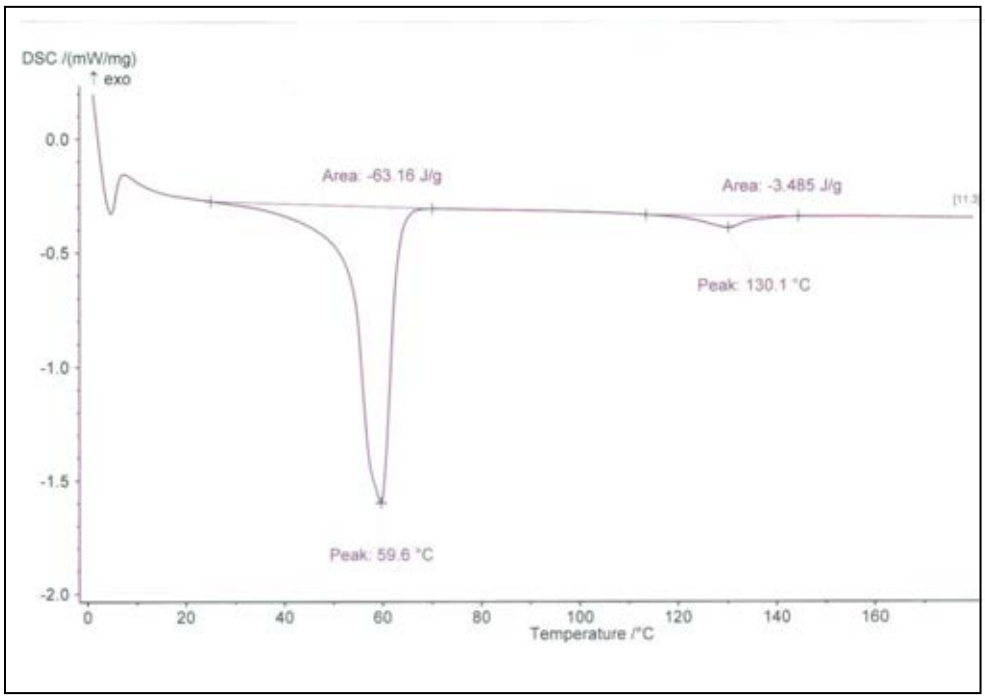


Figure A23 DSC chromatogram of CHI/PLA/PCL blend films at the ratios of 60:4:36

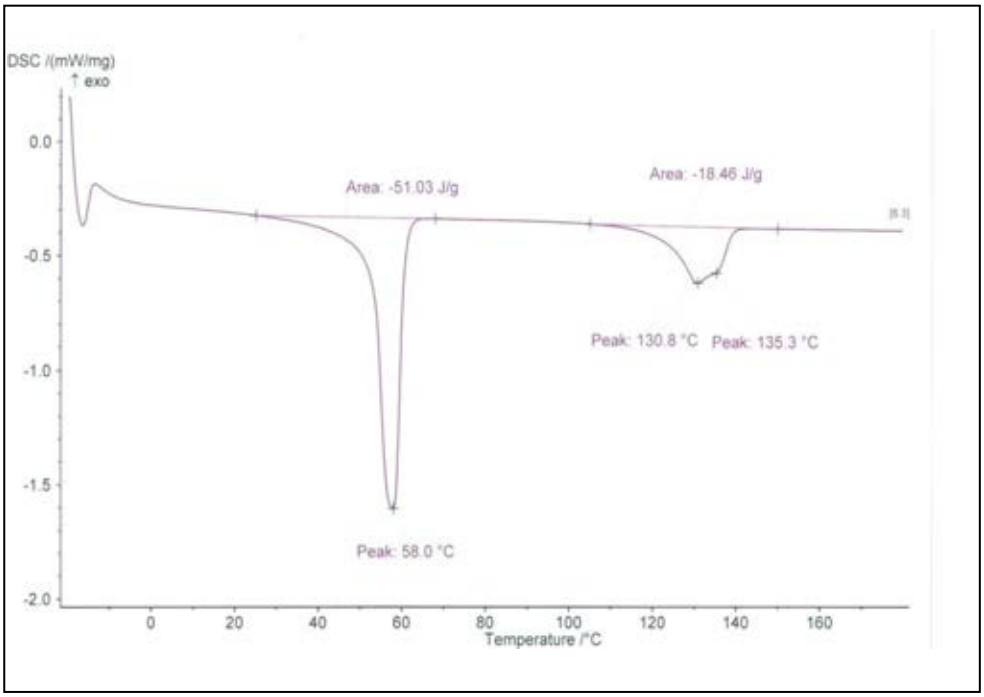


Figure A24 DSC chromatogram of CHI/PLA/PCL blend films at the ratios of 60:12:28

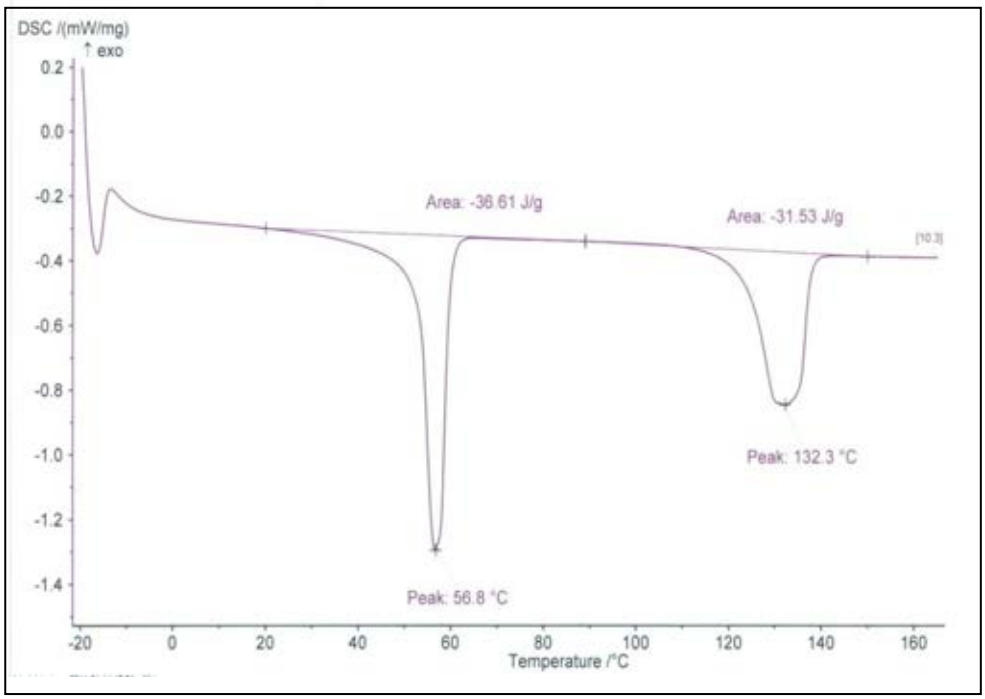


Figure A25 DSC chromatogram of CHI/PLA/PCL blend films at the ratios of 60:20:20.

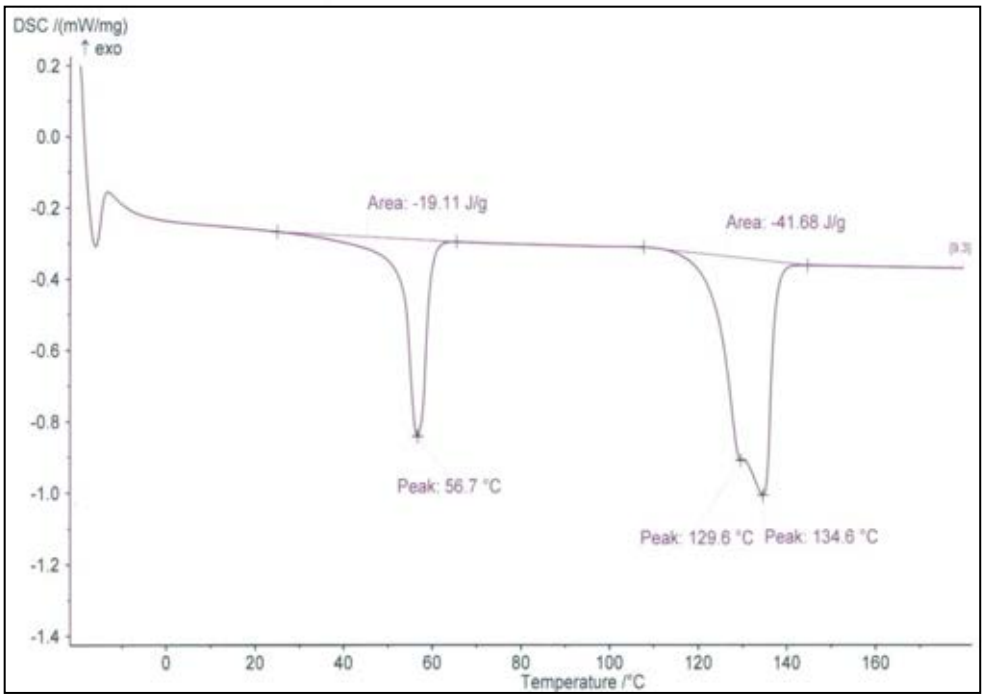


Figure A26 DSC chromatogram of CHI/PLA/PCL blend films at the ratios of 60:28:12.

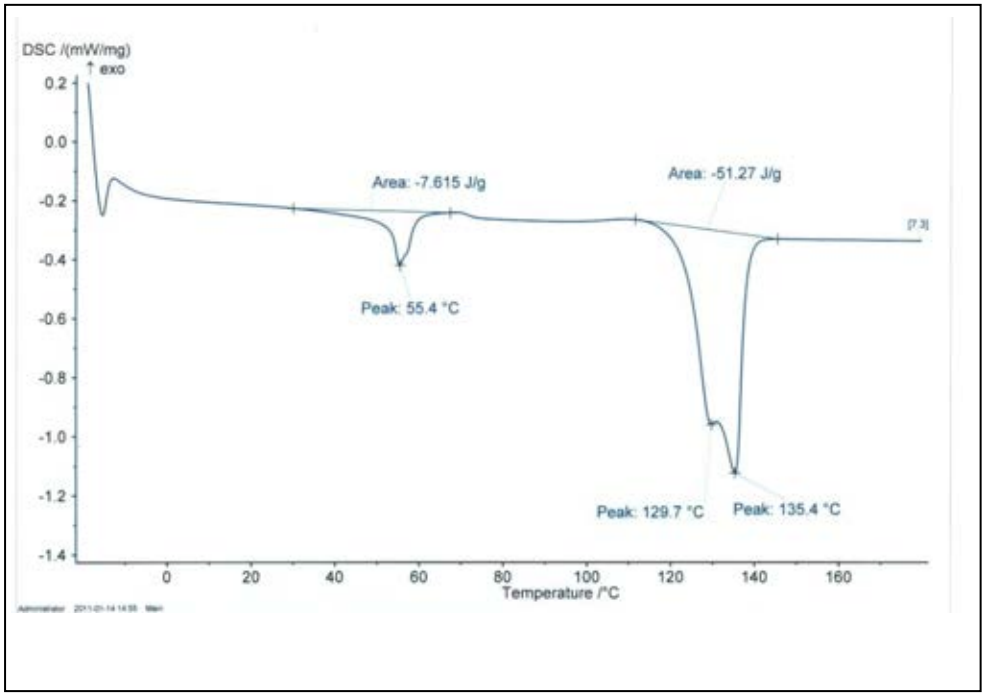


Figure A27 DSC chromatogram of CHI/PLA/PCL blend films at the ratios of 60:36:4.

APPENDIX B

THE DEGREE OF SWELLING

Table B1 Swelling behavior of CHI/PLA/PCL blend films with the presence of Gly at 0.5 wt% with the different PLA and PCL ratios in PBS buffer (pH 7.4). The total film weight was set at 1.0 g.

Time(hr)	Swelling Degree (%)														
	CHI/PLA/PCL (60:4:36)			CHI/PLA/PCL (60:12:28)			CHI/PLA/PCL (60:20:20)			CHI/PLA/PCL (60:28:12)			CHI/PLA/PCL (60:36:4)		
0.000	0	±	0	0	±	0	0	±	0	0	±	0	0	±	0
0.017	397.04	±	6.80	205.30	±	8.34	291.00	±	8.60	132.94	±	6.74	139.66	±	8.72
0.033	668.31	±	7.32	463.55	±	14.23	363.21	±	31.94	334.90	±	36.64	307.84	±	5.90
0.050	762.61	±	82.25	650.31	±	8.03	574.31	±	44.65	469.34	±	24.03	467.52	±	18.84
0.067	782.07	±	70.79	759.33	±	7.19	645.82	±	43.03	598.99	±	22.43	544.17	±	15.70
0.083	826.89	±	86.90	853.98	±	28.09	720.96	±	2.82	681.63	±	34.99	660.59	±	48.45
0.100	894.48	±	100.14	898.24	±	5.25	755.41	±	20.51	745.08	±	37.95	729.68	±	49.89
0.117	907.10	±	85.29	920.11	±	6.71	813.38	±	17.75	783.92	±	45.56	782.78	±	23.96
0.133	919.66	±	79.10	933.57	±	13.24	879.77	±	24.36	817.79	±	37.70	828.77	±	30.87
0.150	926.52	±	84.77	951.71	±	18.71	893.00	±	29.22	843.81	±	30.81	863.59	±	32.36
0.167	935.93	±	78.14	967.30	±	33.52	922.41	±	18.26	867.49	±	29.73	880.02	±	30.18
0.250	944.50	±	76.39	953.21	±	12.06	959.41	±	19.86	937.24	±	33.87	930.39	±	41.87
0.333	957.73	±	69.99	968.76	±	10.37	981.42	±	27.55	949.60	±	26.59	960.33	±	57.84
0.417	965.33	±	66.00	973.87	±	6.66	991.07	±	29.34	959.49	±	31.28	981.71	±	54.87
0.500	968.18	±	61.17	982.85	±	8.30	998.09	±	27.12	977.91	±	26.53	1005.87	±	41.04
0.583	973.84	±	56.07	990.76	±	8.04	1007.96	±	20.31	984.69	±	28.46	1014.56	±	22.97
0.750	980.35	±	54.14	1000.09	±	5.45	1019.18	±	15.85	991.87	±	24.05	1025.13	±	20.60
1.000	991.15	±	52.83	1007.12	±	1.84	1029.44	±	15.64	1004.65	±	22.51	1036.96	±	13.28

APPENDIX C

CALIBRATION CURVE OF DOXYCYCLINE

Calibration curve of doxycycline

The concentration versus absorbance of doxycycline in phosphate buffer saline pH 7.4 at 74 nm is presented in Table C1. The standard curve of doxycycline in these solution media is illustrated in Figure C1.

Table C1 Absorbance of doxycycline in PBS at 274 nm.

Concentration (mg/L)	Absorbance
5	0.155
10	0.292
20	0.563
30	0.867
40	1.144
50	1.421

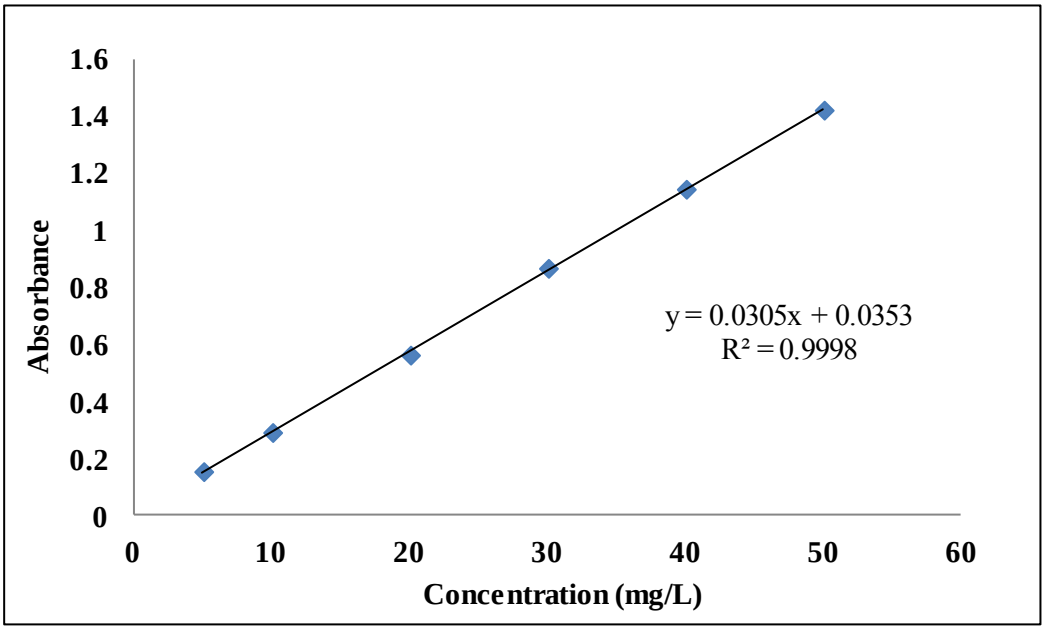


Figure C1 Calibration curve of doxycycline in PBS at 274 nm.

APPENDIX D

PERCENTAGE OF DRUG RELEASE

Table D1 Percentage of doxycycline from CHI/PLA/PCL blend film (CHI/PLA/PCL 60:4:36) in PBS at 37°C.

Time (h)	%Doxycycline released
0.00	0 ± 0
0.04	60.51 ± 4.36
0.08	73.19 ± 1.24
0.13	70.04 ± 2.11
0.17	72.56 ± 2.37
0.21	75.19 ± 0.74
0.25	77.97 ± 1.00
0.29	79.63 ± 1.47
0.33	79.66 ± 3.98
0.38	77.55 ± 3.72
0.42	78.49 ± 4.15
0.46	81.46 ± 6.79
0.50	80.82 ± 6.16
0.58	82.05 ± 7.08
0.67	81.78 ± 4.53
0.75	82.00 ± 3.53
0.83	83.54 ± 3.08
1.00	85.43 ± 3.66
1.25	86.80 ± 3.32
1.50	87.28 ± 4.01
1.75	86.74 ± 4.37
2.00	85.79 ± 3.63

Table D2 Percentage of doxycycline from CHI/PLA/PCL blend film (CHI/PLA/PCL 60:12:28) in PBS at 37°C.

Time (h)	%Doxycycline released
0.00	0 ± 0
0.04	54.92 ± 2.68
0.08	77.80 ± 5.01
0.13	78.47 ± 4.54
0.17	79.59 ± 3.70
0.21	80.41 ± 2.11
0.25	83.33 ± 3.46
0.29	82.64 ± 1.78
0.33	80.47 ± 1.47
0.38	79.86 ± 1.18
0.42	81.43 ± 1.89
0.46	81.93 ± 2.88
0.50	82.98 ± 2.87
0.58	84.54 ± 3.22
0.67	84.67 ± 3.36
0.75	85.16 ± 2.61
0.83	83.84 ± 2.96
1.00	85.74 ± 2.92
1.25	84.95 ± 2.80
1.50	85.49 ± 2.13
1.75	85.68 ± 2.59
2.00	85.09 ± 2.57

Table D3 Percentage of doxycycline from CHI/PLA/PCL blend film (CHI/PLA/PCL 60:20:20) in PBS at 37°C.

Time (h)	%Doxycycline released
0.00	0 ± 0
0.04	50.17 ± 4.74
0.08	62.12 ± 1.84
0.13	71.49 ± 2.26
0.17	73.16 ± 2.58
0.21	73.45 ± 2.93
0.25	74.04 ± 4.89
0.29	75.20 ± 5.12
0.33	75.25 ± 6.15
0.38	74.42 ± 5.39
0.42	75.17 ± 4.34
0.46	76.61 ± 4.39
0.50	78.37 ± 5.34
0.58	78.54 ± 5.27
0.67	80.00 ± 4.52
0.75	80.65 ± 4.54
0.83	80.19 ± 5.40
1.00	81.08 ± 4.87
1.25	80.83 ± 5.94
1.50	80.61 ± 6.50
1.75	80.83 ± 5.99
2.00	80.68 ± 6.81

Table D4 Percentage of doxycycline from CHI/PLA/PCL blend film (CHI/PLA/PCL 60: 28:12) in PBS at 37°C.

Time (h)	%Doxycycline released
0.00	0 ± 0
0.04	60.90 ± 1.99
0.08	76.82 ± 1.79
0.13	75.68 ± 1.63
0.17	79.23 ± 1.57
0.21	82.28 ± 2.87
0.25	82.62 ± 3.05
0.29	84.08 ± 1.25
0.33	83.19 ± 2.81
0.38	83.27 ± 3.75
0.42	85.25 ± 3.45
0.46	85.73 ± 2.74
0.50	85.40 ± 4.55
0.58	88.35 ± 3.37
0.67	88.19 ± 2.85
0.75	89.79 ± 2.72
0.83	88.59 ± 3.79
1.00	87.27 ± 4.35
1.25	88.09 ± 3.86
1.50	88.56 ± 3.33
1.75	86.27 ± 1.52
2.00	87.48 ± 1.52

Table D5 Percentage of doxycycline from CHI/PLA/PCL blend film (CHI/PLA/PCL 60:4:36) in PBS at 37°C.

Time (h)	%Doxycycline released
0.00	0 ± 0
0.04	52.82 ± 1.62
0.08	75.40 ± 4.10
0.13	73.98 ± 3.80
0.17	76.97 ± 2.89
0.21	78.71 ± 2.86
0.25	79.75 ± 4.05
0.29	82.70 ± 5.42
0.33	83.39 ± 6.54
0.38	85.07 ± 6.78
0.42	83.93 ± 7.22
0.46	87.02 ± 7.18
0.50	87.04 ± 6.98
0.58	86.64 ± 5.01
0.67	87.12 ± 5.54
0.75	87.97 ± 5.27
0.83	88.41 ± 5.12
1.00	86.91 ± 4.83
1.25	87.37 ± 3.60
1.50	86.18 ± 2.90
1.75	86.51 ± 3.34
2.00	86.02 ± 3.30

Table D6 Percentage of MSG and doxycycline from MSG/ doxycycline loaded CHI/PLA/PCL blend film in PBS at 37°C.

Time (h)	% released	
	MSG	**Doxycycline**
0.00	0 ± 0	0 ± 0
0.04	75.24 ± 2.61	35.02 ± 2.53
0.08	80.36 ± 4.16	39.40 ± 2.33
0.13	84.52 ± 4.49	42.09 ± 3.07
0.17	87.55 ± 3.03	44.31 ± 1.57
0.21	86.39 ± 2.40	47.24 ± 2.29
0.25	89.86 ± 3.84	51.20 ± 2.25
0.29	91.33 ± 4.85	54.18 ± 2.09
0.33	91.20 ± 4.15	57.49 ± 2.07
0.38	91.97 ± 4.99	60.43 ± 2.39
0.42	91.79 ± 5.57	62.72 ± 1.51
0.46	92.33 ± 5.14	65.11 ± 0.50
0.50	93.31 ± 3.82	66.60 ± 1.04
0.58	93.76 ± 3.86	68.63 ± 1.60
0.67	93.61 ± 3.39	70.17 ± 2.71
0.75	94.70 ± 3.01	72.30 ± 3.23
0.83	95.74 ± 2.71	73.76 ± 3.38
1.00	96.33 ± 2.07	74.91 ± 4.58
1.25	97.01 ± 1.67	77.21 ± 4.74
1.50	97.25 ± 1.38	77.40 ± 4.87
1.75	97.34 ± 1.49	78.73 ± 4.94
2.00	98.12 ± 1.14	80.01 ± 5.13

APPENDIX E

BLOOD CLOTTING TEST

Table E1 Effect of monosodium glutamate model solution on PT and aPTT.

Concentration (g/L)	Fibrin formation	PT(s)	aPTT(s)
Blood plasma		14.45 ± 0.37	30.89 ± 0.83
10	–	13.40 ± 0.14	25.41 ± 0.37
35	–	11.04 ± 0.95	20.08 ± 0.36
50	–	14.75 ± 0.78	41.97 ± 0.67
100	–	18.28 ± 0.81	46.38 ± 0.92
200	+	46.91 ± 1.01	101.75 ± 0.75
400	+	N.D.	N.D.
Saturated	+	N.D.	N.D.

n.d.: not determinable because statistical coagulation tests were not occurred.

APPENDIX F

ANTIBACTERIAL ACTIVITY TEST

Table F1 OD_{600} of *S. aureus* when different polymeric films were immersed into the bacterial culture broth compared with the control bacterial culture where no film was present.

Time (h)	O.D.(abs.)													
	control		Chitosan		N.Chitosan		PLA		CHI/PLA/PCL		Commercial		Doxycycline	
0	0.073	± 0.020	0.074	± 0.017	0.070	± 0.013	0.072	± 0.005	0.074	± 0.002	0.073	± 0.000	0.060	± 0.002
3	2.393	± 0.302	0.144	± 0.026	2.188	± 1.573	2.327	± 0.225	1.667	± 0.125	2.010	± 0.151	0.086	± 0.004
6	5.963	± 1.040	0.152	± 0.022	7.027	± 1.325	7.657	± 0.282	5.933	± 0.076	6.577	± 1.202	0.137	± 0.003
9	11.177	± 2.186	0.203	± 0.011	8.653	± 0.697	11.437	± 1.701	9.647	± 0.432	11.643	± 1.179	0.129	± 0.007
12	15.300	± 2.476	3.943	± 0.180	19.367	± 1.888	17.667	± 0.551	13.400	± 0.529	14.657	± 1.766	0.119	± 0.009
15	19.800	± 1.500	4.753	± 0.444	18.967	± 0.896	21.767	± 1.301	20.650	± 0.786	19.450	± 1.903	0.133	± 0.005
18	19.200	± 2.500	7.100	± 1.398	19.533	± 1.185	20.333	± 1.724	21.547	± 0.885	20.043	± 0.581	0.135	± 0.002
21	19.200	± 0.700	18.167	± 0.874	19.433	± 0.839	20.567	± 1.940	21.410	± 1.100	19.670	± 0.754	0.139	± 0.003
24	19.900	± 1.852	19.800	± 0.624	17.867	± 1.021	19.867	± 0.907	23.467	± 0.666	19.797	± 0.435	0.135	± 0.004

Table F2 Cell concentration of *S. aureus* when different polymeric films were immersed into the bacterial culture broth compared with the control bacterial culture where no film was present.

Time (h)	Cell concentration(g/L)													
	control		Chitosan		N.Chitosan		PLA		CHI/PLA/PCL		Commercial		Doxycycline	
0	0.52	± 0.06	0.52	± 0.05	0.51	± 0.04	0.51	± 0.01	0.52	± 0.01	0.52	± 0.00	0.48	± 0.07
3	2.41	± 0.11	1.55	± 0.46	2.25	± 0.72	1.99	± 0.36	2.23	± 0.30	2.17	± 0.12	1.77	± 0.20
6	3.59	± 0.35	1.67	± 0.35	2.71	± 0.87	2.59	± 0.39	3.28	± 0.30	2.54	± 0.20	2.00	± 0.03
9	3.70	± 0.73	2.44	± 0.31	3.85	± 0.71	3.29	± 0.17	3.37	± 0.25	2.96	± 0.18	2.43	± 0.11
12	4.29	± 0.70	2.83	± 0.34	4.24	± 0.24	3.98	± 0.09	3.98	± 0.29	3.61	± 0.14	2.29	± 0.37
15	4.67	± 0.78	3.27	± 0.42	4.56	± 0.32	4.04	± 0.88	4.78	± 0.44	4.79	± 0.62	2.09	± 0.35
18	5.06	± 0.47	3.65	± 0.29	4.77	± 0.38	5.03	± 0.25	4.84	± 0.48	4.90	± 0.17	2.31	± 0.28
21	4.82	± 0.51	4.42	± 0.58	4.95	± 0.29	4.95	± 0.14	5.03	± 0.22	5.26	± 0.18	2.29	± 0.28
24	5.01	± 0.71	4.35	± 0.67	5.47	± 0.33	5.18	± 0.45	5.28	± 0.70	5.37	± 0.14	2.24	± 0.36

Table F3 Log (CFU/mL) of *S. aureus* when different polymeric films were immersed into the bacterial culture broth compared with the control bacterial culture where no film was present.

Time (h)	Log (CFU/mL)						
	control	Chitosan	N.Chitosan	PLA	CHI/PLA/PCL	Commercial	Doxycycline
0	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000
3	9.42 ± 0.415	4.63 ± 0.966	7.65 ± 0.864	9.70 ± 0.553	8.19 ± 0.444	9.49 ± 1.069	5.34 ± 0.226
6	11.93 ± 1.249	4.73 ± 1.169	10.36 ± 0.539	13.43 ± 0.295	10.56 ± 0.068	12.55 ± 0.983	5.13 ± 0.620
9	12.61 ± 2.002	6.00 ± 0.568	10.43 ± 0.514	14.49 ± 0.168	11.06 ± 0.499	13.22 ± 1.090	4.56 ± 0.403
12	12.12 ± 0.684	8.68 ± 1.853	11.80 ± 0.254	14.73 ± 0.652	11.72 ± 0.463	13.46 ± 1.299	5.84 ± 0.185
15	13.15 ± 0.627	12.04 ± 1.889	13.66 ± 0.554	14.92 ± 1.032	13.43 ± 0.665	13.44 ± 0.791	5.50 ± 0.031
18	12.91 ± 1.597	12.41 ± 1.788	12.90 ± 0.579	14.46 ± 0.742	13.94 ± 0.050	13.63 ± 0.930	5.76 ± 0.113
21	11.97 ± 1.325	12.59 ± 0.229	13.27 ± 0.836	14.69 ± 0.977	13.62 ± 0.237	13.69 ± 1.171	6.05 ± 2.108
24	11.83 ± 1.233	12.18 ± 0.337	11.95 ± 0.916	13.91 ± 0.719	12.79 ± 0.641	13.71 ± 1.112	5.27 ± 0.577

Table F4 OD_{600} of *E.coli* when different polymeric films were immersed into the bacterial culture broth compared with the control bacterial culture where no film was present.

time (hr.)	O.D.(abs.)						
	control	**Chitosan**	**N.Chitosan**	**PLA**	**CHI/PLA/PCL**	**Commercial**	**Doxycycline**
0	0.079 ± 0.012	0.042 ± 0.025	0.092 ± 0.000	0.066 ± 0.013	0.043 ± 0.013	0.028 ± 0.000	0.051 ± 0.000
3	3.830 ± 1.420	2.437 ± 0.264	4.270 ± 0.154	2.623 ± 0.430	2.540 ± 0.113	2.227 ± 0.150	0.123 ± 0.033
6	7.690 ± 0.579	5.500 ± 1.373	7.480 ± 0.271	7.817 ± 0.841	9.400 ± 0.531	7.903 ± 0.206	0.156 ± 0.014
9	9.653 ± 0.119	7.517 ± 0.732	9.407 ± 0.321	9.113 ± 0.110	9.860 ± 0.317	9.700 ± 0.046	0.153 ± 0.018
12	9.073 ± 0.142	8.717 ± 0.679	10.810 ± 0.226	9.297 ± 0.259	10.000 ± 0.300	9.497 ± 0.179	0.134 ± 0.018
15	9.100 ± 0.539	8.410 ± 0.450	8.433 ± 0.220	9.277 ± 0.340	9.727 ± 0.660	8.763 ± 0.115	0.152 ± 0.013
18	8.873 ± 0.286	7.930 ± 0.223	8.817 ± 0.227	8.917 ± 0.232	8.873 ± 0.520	9.003 ± 0.110	0.161 ± 0.023
21	8.210 ± 0.053	7.690 ± 0.269	9.260 ± 0.165	8.690 ± 0.364	8.903 ± 0.716	9.113 ± 0.061	0.148 ± 0.012
24	7.827 ± 0.528	7.777 ± 0.407	8.583 ± 0.176	8.503 ± 0.616	8.750 ± 0.302	8.637 ± 0.569	0.163 ± 0.022

Table F5 Cell concentration(g/L) of *E.coli* when different polymeric films were immersed into the bacterial culture broth compared with the control bacterial culture where no film was present.

time (hr.)	Cell concentration(g/L)																							
	control			Chitosan			N.Chitosan			PLA			CHI/PLA/PCL			Commercial			Doxycycline		
0	0.54	±	0.02	0.47	±	0.05	0.57	±	0.00	0.52	±	0.52	0.47	±	0.03	0.44	±	0.00	0.49	±	0.00
3	3.63	±	0.48	1.57	±	0.25	3.31	±	0.08	3.13	±	0.69	2.89	±	0.06	2.24	±	0.39	1.05	±	0.06
6	3.49	±	0.54	2.70	±	0.18	3.55	±	0.31	3.23	±	0.63	3.12	±	0.19	3.07	±	0.13	1.10	±	0.35
9	3.22	±	0.63	2.77	±	0.36	3.60	±	0.16	3.71	±	0.96	3.11	±	0.32	3.64	±	0.25	2.19	±	0.09
12	3.23	±	0.24	2.70	±	0.34	3.77	±	0.09	3.11	±	0.38	3.26	±	0.31	3.55	±	0.14	2.08	±	0.05
15	3.71	±	0.79	3.10	±	0.08	3.37	±	0.28	3.25	±	0.20	3.38	±	0.13	3.67	±	0.10	2.11	±	0.06
18	3.17	±	0.27	3.24	±	0.29	3.68	±	0.07	3.54	±	0.34	3.34	±	0.34	3.69	±	0.16	2.08	±	0.02
21	3.45	±	0.85	3.19	±	0.20	3.76	±	0.07	3.87	±	0.21	3.31	±	0.14	3.54	±	0.15	2.13	±	0.08
24	3.60	±	0.33	3.31	±	0.21	3.77	±	0.06	3.67	±	0.07	3.35	±	0.23	3.62	±	0.10	2.36	±	0.11

Tabe F6 Log (CFU/mL) of *E.coli* when different polymeric films were immersed into the bacterial culture broth compared with the control bacterial culture where no film was present.

time (hr.)	Log (CFU/mL)							
	control	**Chitosan**	**N.Chitosan**	**PLA**	**CHI/PLA/PCL**	**Commercial**	**Doxycycline**
0	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000	1.00 ± 0.000
3	11.24 ± 0.321	9.02 ± 0.544	9.54 ± 0.396	9.48 ± 0.239	9.48 ± 0.239	9.17 ± 0.346	6.26 ± 0.692
6	11.85 ± 0.197	12.03 ± 0.569	11.33 ± 0.969	12.12 ± 0.511	12.12 ± 0.511	11.23 ± 0.656	6.74 ± 0.503
9	12.17 ± 0.727	12.25 ± 0.622	12.78 ± 0.728	12.46 ± 0.414	12.46 ± 0.414	12.55 ± 0.045	8.18 ± 1.129
12	12.43 ± 1.182	12.26 ± 0.095	12.74 ± 0.051	12.72 ± 0.576	12.72 ± 0.576	13.14 ± 0.746	7.50 ± 0.213
15	12.58 ± 0.533	12.64 ± 0.126	12.85 ± 0.648	13.05 ± 0.210	13.05 ± 0.210	12.77 ± 0.229	7.50 ± 0.245
18	12.65 ± 0.143	13.06 ± 0.635	12.74 ± 0.723	13.30 ± 0.340	13.30 ± 0.340	12.86 ± 0.771	7.29 ± 0.196
21	12.36 ± 0.216	13.45 ± 0.650	13.53 ± 0.512	13.04 ± 0.785	13.04 ± 0.785	12.93 ± 0.603	7.28 ± 0.088
24	12.51 ± 0.146	13.59 ± 0.103	13.33 ± 0.844	13.51 ± 0.691	13.51 ± 0.691	12.53 ± 0.112	7.61 ± 0.120

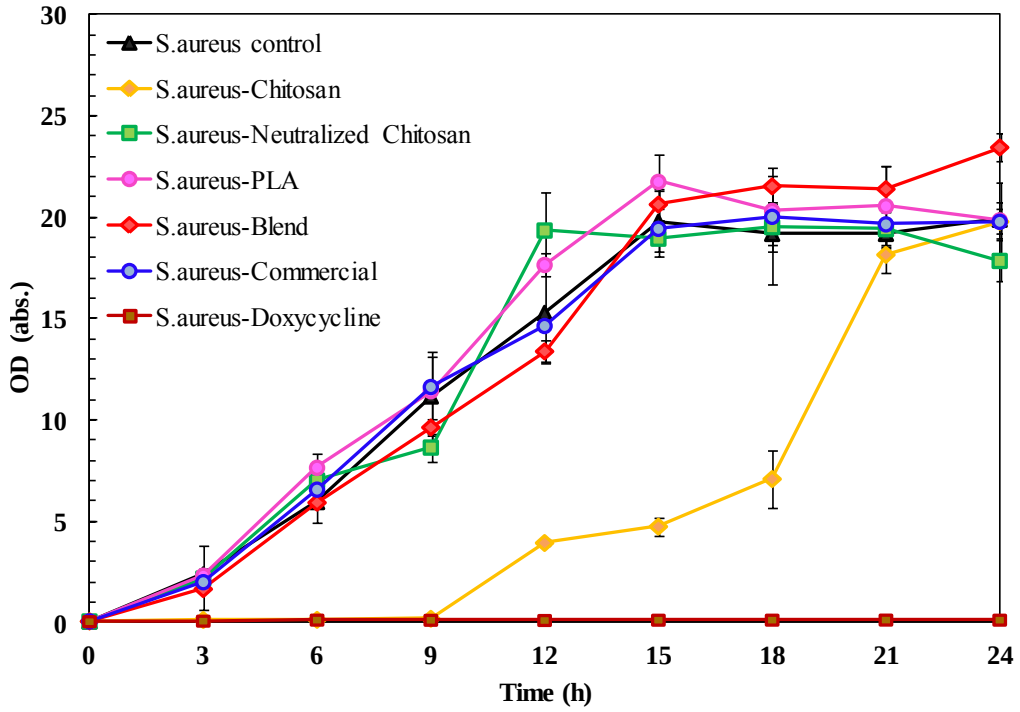


Figure F7 Optical densities (OD_{600}) of *S.aureus in* media alone (control) and in the presence of different materials.

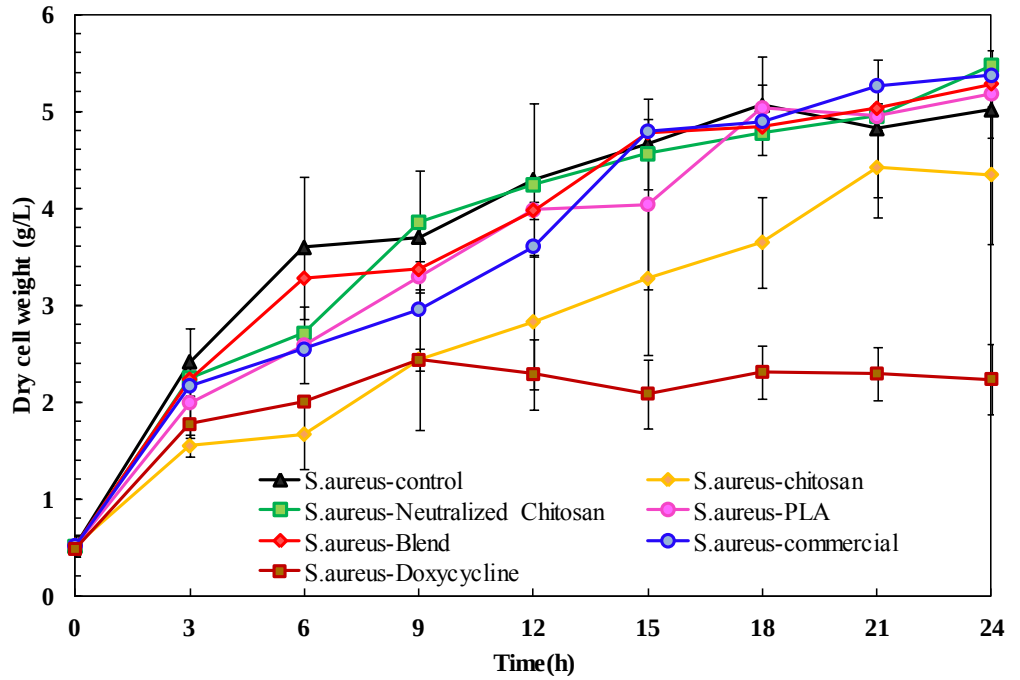


Figure F8 Dry cell weight of *S.aureus* in media alone (control) and in the presence of different materials

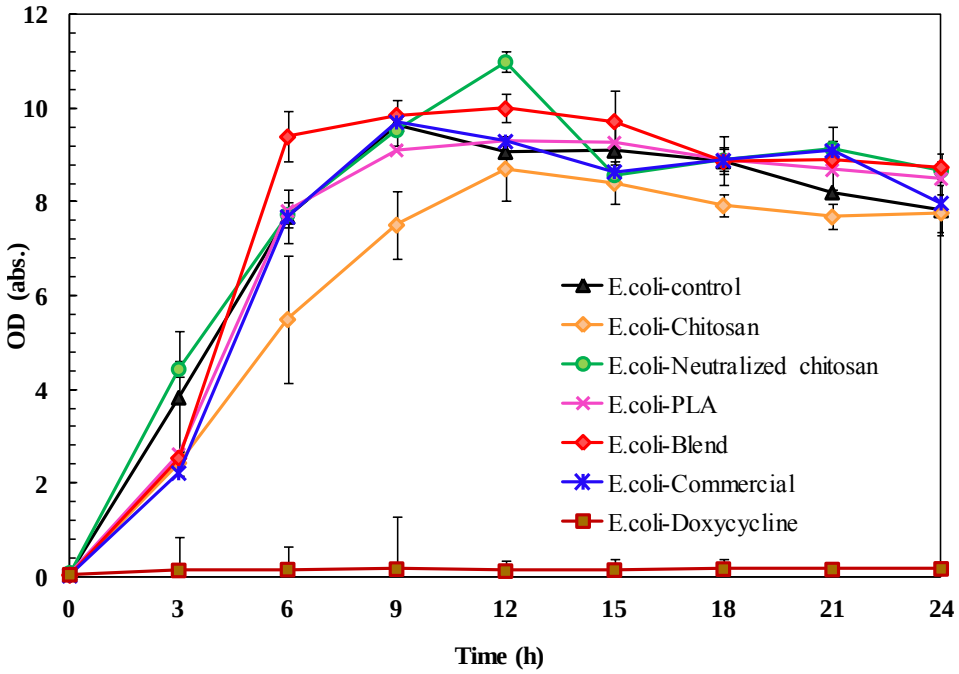


Figure F9 Optical densities (OD_{600}) of *E.coli* in media alone (control) and in the presence of different materials.

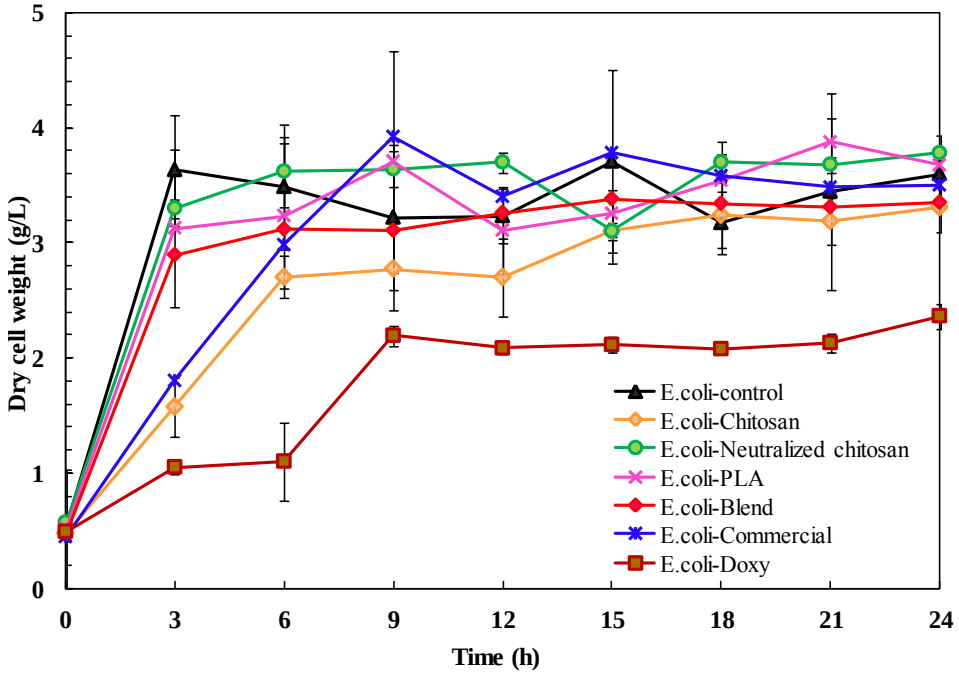


Figure F10 Dry cell weight of *E.coli* in media alone (control) and in the presence of different materials.

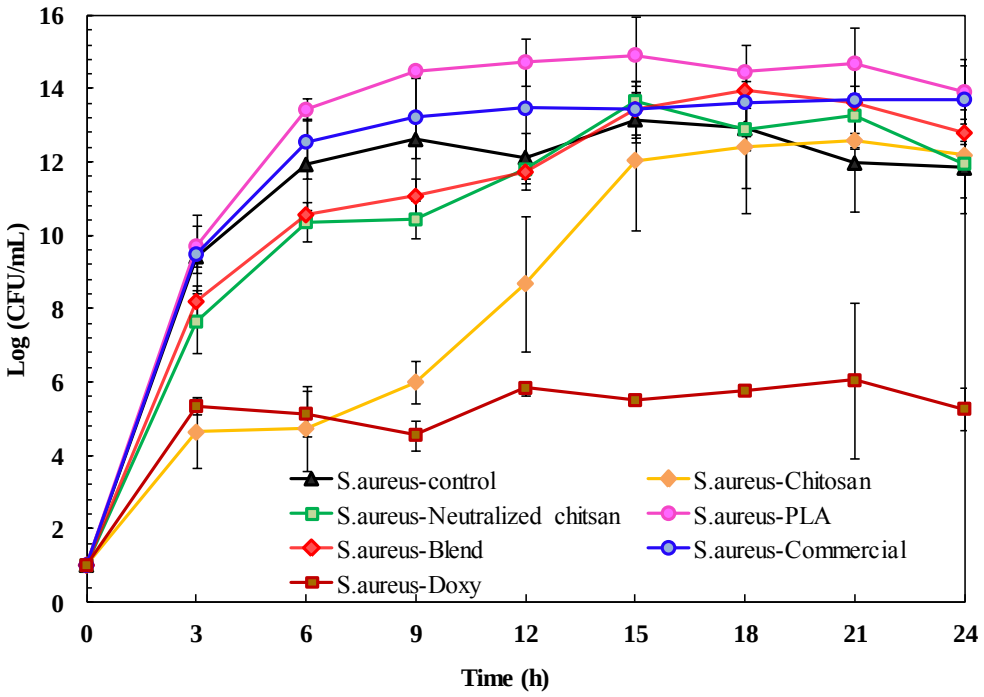


Figure F11 Total replication competent (viable) cell counts of bacteria grown for 24 h in media alone (control) and in the presence of different materials.

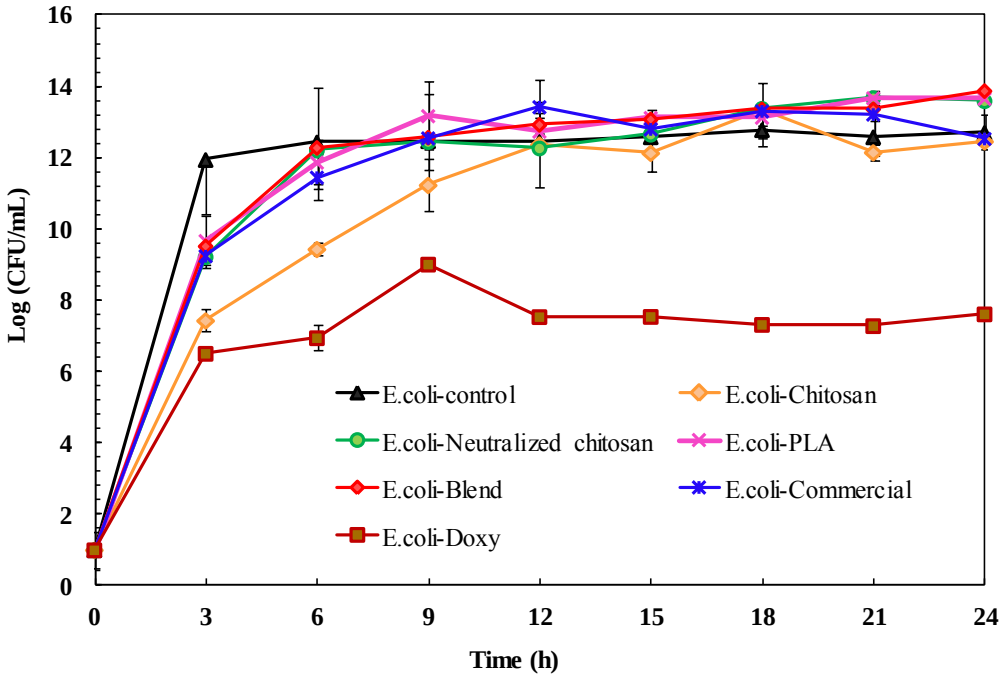


Figure F12 Total replication competent (viable) cell counts of bacteria grown for 24 h in media alone (control) and in the presence of different materials

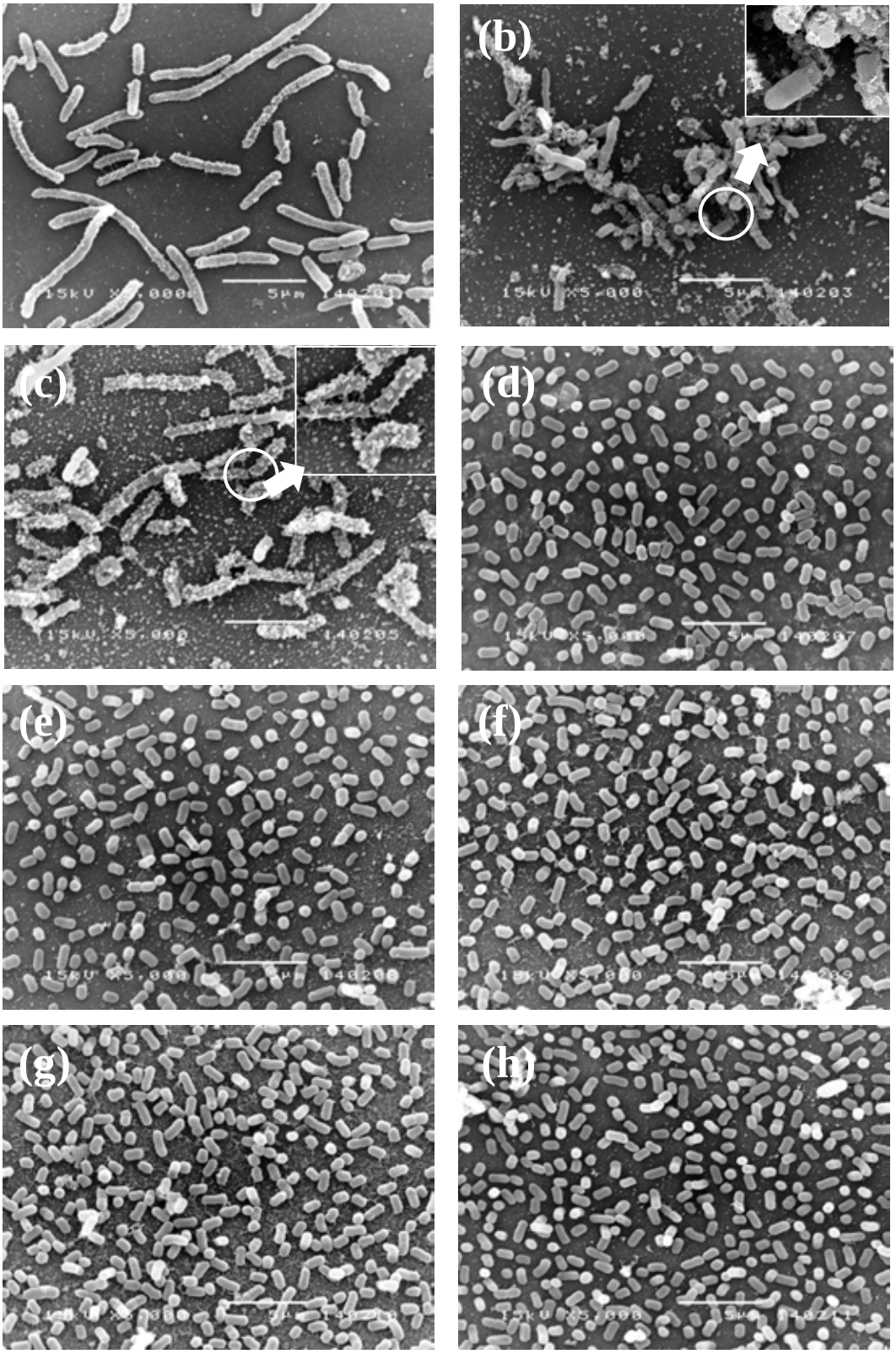


Figure F13 Scanning electron micrographs of chitosan film after being exposed to *E.coli* (×5,000) in various times (a) 3 h., (b) 6 h., (c) 9 h., (d) 12 h., (e) 15 h., (f) 18 h., (g) 21 h., and (h) 24 h.

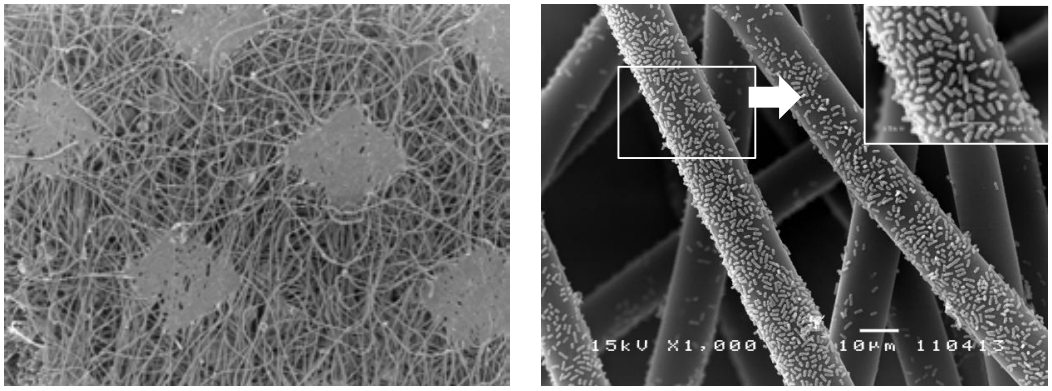


Figure F14 Scanning electron micrographs of Boots stop bleeding fast dressing after being exposed to *E.coli* (×5,000) in 3 h.

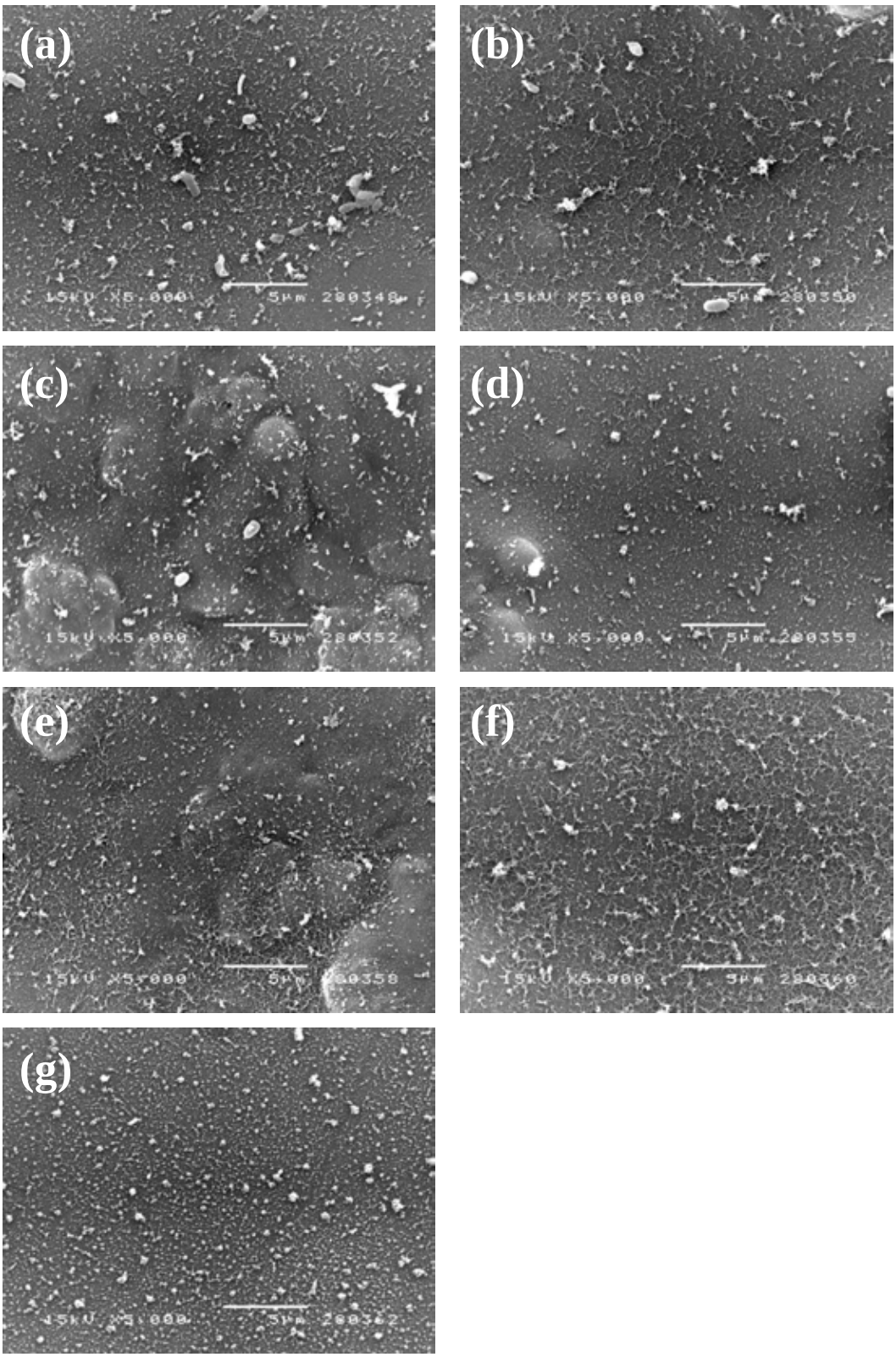


Figure F15 Scanning electron micrographs of doxycycline loaded blend film after being exposed to *E.coli* (×5,000) in various times (a) 3 h., (b) 6 h., (c) 9 h., (d) 12 h., (e) 15 h., (f) 18 h., (g) 21 h., and (h)

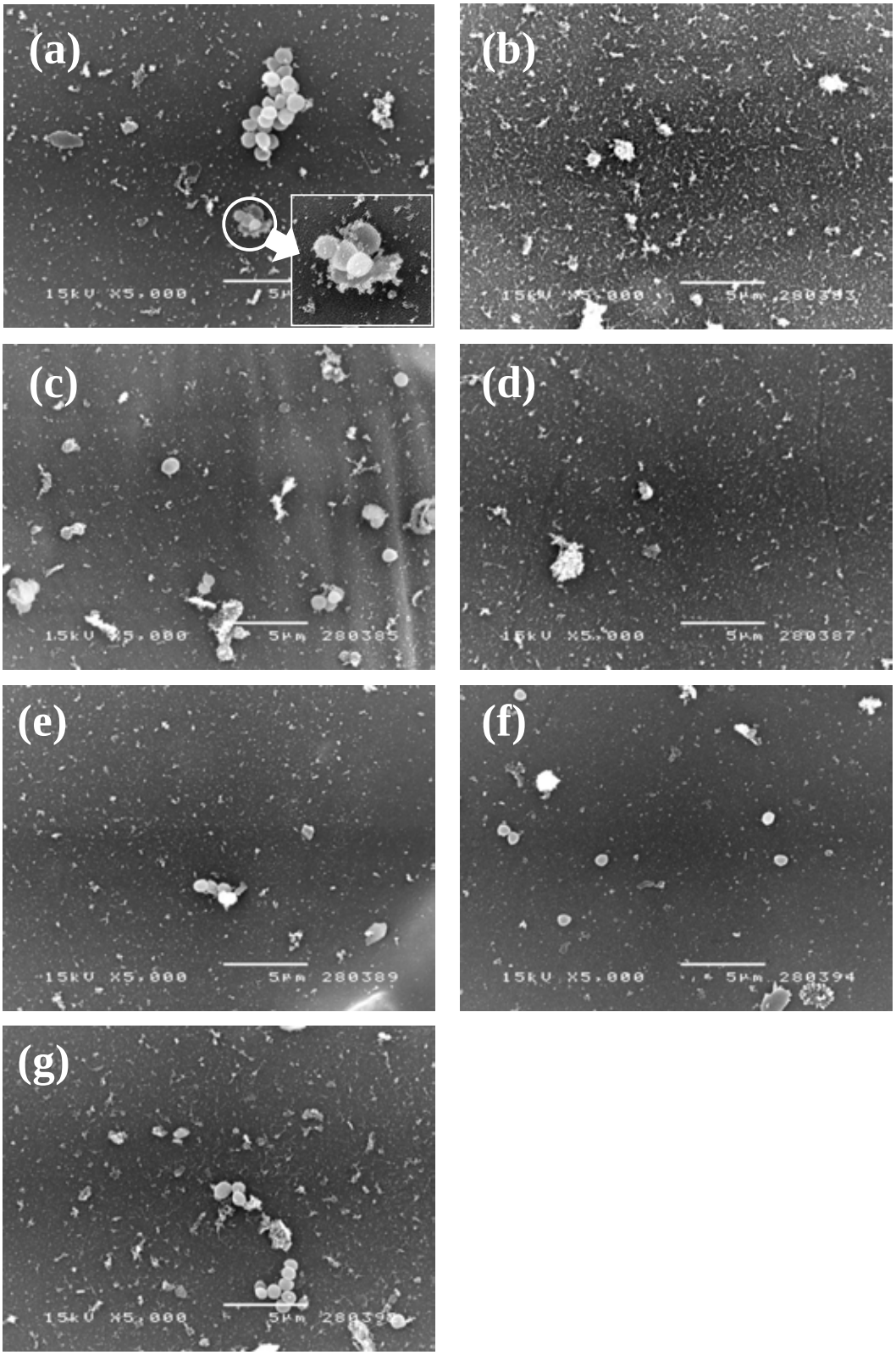


Figure F16 Scanning electron micrographs of doxycycline loaded blend film after being exposed to *S.aureus* (×5,000) in various times (a) 3 h., (b) 6 h., (c) 9 h., (d) 12 h., (e) 15 h., (f) 18 h., (g) 21 h., and (h) 24 h

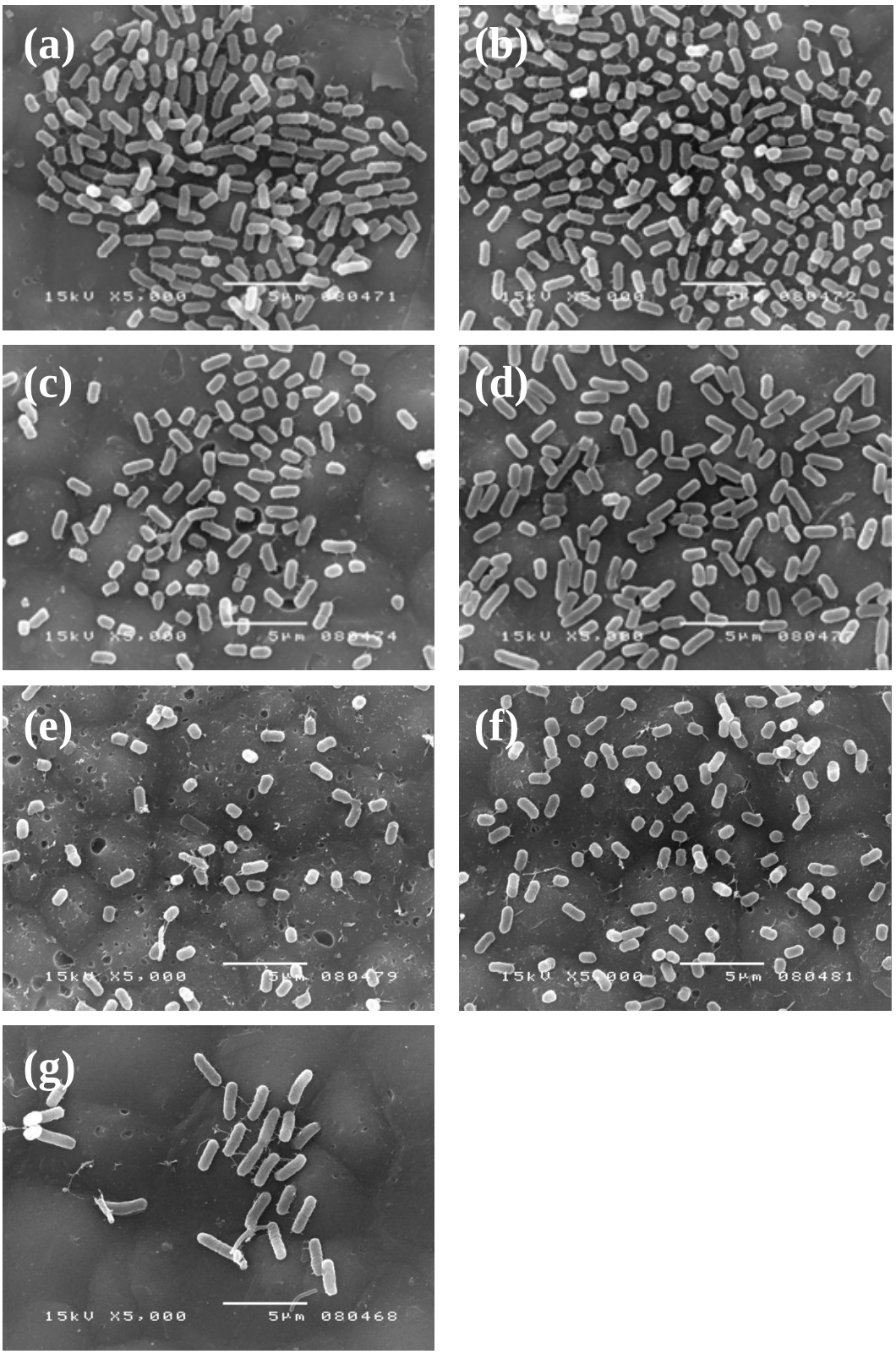


Figure F17 Scanning electron micrographs of PLA film after being exposed to *E.coli* (×5,000) in various times (a) 3 h., (b) 6 h., (c) 9 h., (d) 12 h., (e) 15 h., (f) 18 h., (g) 21 h., and (h)

VITA

Ms. Wasinee Boonkong was born on Wednesday 19th August, 1981, in Chachoengsao, Thailand. In 2004, she graduated with a Bachelor's degree of Science in Chemistry, from Chulalongkorn University. After that she pursued her master in the program of Petrochemical and polymer science in Faculty of Science, Chulalongkorn University, and completed her requirements in 2006. In 2007, she began her doctoral degree in Petrochemistry, Faculty of Science, Chulalongkorn University. During her doctoral study, she was granted by the 90th anniversary of Chulalongkorn University Fund for financed support throughout her study. She is projected to complete her doctoral degree in 2012.